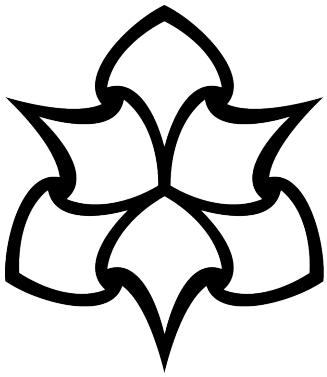

Risk Stratification for Thrombosis in Type 2 Diabetic Patients

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Abstract

The leading cause of mortality in type 2 diabetes mellitus patients is cardiovascular disease, with patients exhibiting platelet hyperreactivity and prothrombotic propensity. The mechanisms that underpin these effects however are unclear. The aim of this study was to determine which of the biochemical features associated with type 2 diabetes are responsible for increased platelet reactivity.

Whole blood from fasted healthy participants supplemented with increasing concentrations of glucose, exhibited a significant elevation in mean platelet volume (MPV) and basal $\alpha_{IIb}\beta_3$ receptor activation. Platelet aggregation in response to ADP and collagen was not increased by acute hyperglycaemia in platelet rich plasma or whole blood, and there was no significant increase in α–granule secretion or $\alpha_{IIb}\beta_3$ activation following ADP stimulation. Furthermore, in type 2 diabetic patients, HbA1c and fasting blood glucose correlated with MPV, but not platelet activation or aggregation. This suggests that hyperglycaemia elevates MPV and increases $\alpha_{IIb}\beta_3$ activation in circulating platelets, but does not increase platelet reactivity as previously reported.

The key finding from this study was the strong positive correlation for low-density lipoprotein cholesterol (LDL-C) and both ADP and collagen-activated platelet aggregation, and this was independent of diabetes. More importantly, patients with LDL-C over 2.0 mmol/L had significantly increased platelet aggregation. Furthermore, LDL-C was associated with elevated platelet production, and

participants with LDL-C over 3.0 mmol/L had significantly increased immature platelet fraction.

This study demonstrates that LDL-C may act as a biomarker to identify type 2 diabetes patients who would benefit from antiplatelet prophylaxis. Additionally, lipid-lowering medication could indirectly provide antithrombotic benefit by reducing LDL-C levels. Moreover, enhanced platelet production mediated by elevated LDL-C is likely responsible for both enhanced platelet reactivity and reduced antiplatelet efficacy in type 2 diabetic patients.

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Declaration

I hereby declare that I have personally undertaken all the work described in this thesis.

Dissemination of study findings

Oral Presentations

‘Risk stratification for thrombosis in type 2 diabetic patients.’ Presented at the Science and Engineering Research Symposium (2017), Manchester Metropolitan University, UK.

‘Why So Sticky?’ Presented as a finalist for the 3 Minute Thesis (3MT®) competition (2018), Manchester Metropolitan University, Manchester, UK.

Poster Presentations

‘Characterisation of the Direct and Indirect Mechanisms that Contribute to Platelet Hyperreactivity in Type 2 Diabetes Mellitus.’ Presented at the Northern Vascular Biology Forum (2016), University of Hull, Hull, UK.

‘Characterisation of the Acute Effects of Hyperglycaemia on Platelet Function.’ Presented at the Diabetes UK Professional Conference (2017), Manchester, UK.

‘Elevated LDL-C is Responsible for Increased Platelet Reactivity in Type 2 Diabetes, not Hyperglycaemia.’ Presented at The Platelet Society’s First Early Careers Meeting (2018), Manchester Metropolitan University, Manchester, UK.

‘Risk Stratification for Thrombosis in Type 2 Diabetic Patients.’ Presented at the European Association for the Study of Diabetes (2018), Berlin, Germany.

Publications (Abstract)

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Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ApoER2'	Apolipoprotein E Receptor 2'
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BMI	Body mass index
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CRP	C-reactive protein
DAG	Diacylglycerol
eNOS	Endothelial nitric oxide synthase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer and Nutrition

ERK	Extracellular signal-regulated kinase
FBG	Fasting blood glucose
FcRγ	Fc receptor γ chain
FITC	Fluorescein
fL	Femtolitre
GLUT	Glucose transporter
Gly-apoB	Glycated- apolipoprotein B
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
Hb	Haemoglobin
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein-cholesterol
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
INF	interferon
IP3	Inositol 1,4,5-trisphosphate
IPC	Immature platelet count
IPF	Immature platelet fraction
IFN	Interferon
IP	Interferon gamma-induced protein
IR	Insulin receptor
K2 EDTA	Dipotassium ethylenediaminetetraacetic acid
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LTA	Light transmission aggregometry

MAP	Multi-analyte profiling
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescent intensity
MIP	Macrophage inflammatory protein
MMU	Manchester Metropolitan University
MPV	Mean platelet volume
MRI	Manchester Royal Infirmary
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIDDM	Non-insulin dependent diabetes mellitus
n-LDL	Native-LDL
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NAD phosphate oxidase
NTPDase	Nucleoside triphosphate diphosphohydrolase
OD	Optical density
Ox-LDL	Oxidised-low-density lipoprotein
PAD	Peripheral artery disease
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor

PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDW	Platelet distribution width
PE	Phycoerythrin
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin I_2
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIS	Patient information sheet
PKB	Protein kinase B
PKC	Protein kinase C
P-LCR	Platelet-large cell ratio
$PLC\gamma_2$	Phospholipase $C\gamma_2$
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sAC	Soluble adenylyl cyclase
SD	Standard deviation
sd-LDL	Small dense LDL
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
sICAM-1	Soluble intercellular adhesion molecule
SMC	Smooth muscle cell

SNP	Sodium Nitroprusside
sP	Soluble P
STZ	Streptozotocin
TNF-α	Tumor necrosis factor-α
TO	Thiazole orange
TRAP	Thrombin receptor-activating peptide
TXA2	Thromboxane A2
UKPDS	United Kingdom Prospective Diabetes Study
VASP	Vasodilator-stimulated phosphoprotein
VAT	Visceral adipose tissue
VCAM-1	Vascular cell adhesion molecule-1
vLDL-C	Very low-density lipoprotein-cholesterol
vWF	von Willebrand factor
WHO	World Health Organisation
WT	Wild type

1 Introduction

1.1 Type 2 Diabetes: Definition and Global Prevalence

Under normal conditions, insulin induces the uptake of glucose into muscle, liver and fat tissue (Nussey and Whitehead, 2001). Diabetes is associated with a reduction in insulin secretion and/or insulin resistance, and so the condition is defined by the elevation of blood glucose (Nussey and Whitehead, 2001). Diabetes UK reports that approximately 3.7 million adults have diabetes in the UK and 90% of them have type 2 diabetes mellitus (Diabetes UK, 2017). Further analysis by Diabetes UK relating to figures on obesity and waist circumference, estimates that 12.3 million people are at risk of type 2 diabetes. According to Hex et al. (2012), the National Health Service (NHS) spends approximately £10 billion on diabetes per year. Therefore, the disease imposes a large economic burden on the NHS and a huge risk to public health.

Type 1 diabetes, is an autoimmune deficiency that leads to β-cell death and the decline of insulin being released. Type 2 diabetes is a metabolic disease, caused by the impairment of insulin-dependent glucose uptake into the cells (insulin resistance) and the relative deficiency of insulin secretion by pancreatic β-cells (Nussey and Whitehead, 2001). Individuals can exhibit insulin resistance and subsequent hyperinsulinemia for more than twenty years before developing the disease (Schneider, 2009a).

The risk of developing type 2 diabetes increases more than two-fold with the degree and duration of abdominal obesity (Freemantle et al., 2008). Ethnicity also plays an important role. The prevalence of type 2 diabetes is up to six times greater in African-Caribbean and up to three times greater in the South Asian

people compared to the white population (Department of Health, 2001). It is also more common in people of Chinese descent. More recently, genome wide association studies have detected over eighty loci related to type 2 diabetic susceptibility, and these loci were primarily identified in large cohorts from specific ethnic groups (Andersen et al., 2016). Other research suggests that the condition may begin in the womb. Prenatal exposure to famine might increase the risk of developing type 2 diabetes later in life (Lumey et al., 2015), and this may be through epigenetic changes (Radford et al., 2014).

The disease is diagnosed by an abnormally high blood glucose level, termed hyperglycaemia (Tuch et al., 2000). Fasting blood glucose (FBG) from normal individuals tends to be in the range of 3.5-5.5 mmol/L (Tuch et al., 2000). However, glucose concentrations vary between different diabetic patients and so the definition of diabetes is subjective (Moebus et al., 2011). The cut off points were chosen in relation to levels of glycaemia associated with specific diabetic complications such as retinopathy. The World Health Organisation (WHO) defined diabetes as having fasting plasma levels greater than 7.0 mmol/L (whole blood is 6.1 mmol/L) and post-prandial blood glucose greater than 11.1 mmol/L (WHO, 2006). The haemoglobin A1c (HbA1c) test gives a better indication of glycaemic control over a longer period of time (Tuch et al., 2000). An HbA1c measurement of 42-47 mmol/mol indicates prediabetes and a measurement $\geq$ 48 mmol/mol indicates diabetes (John, 2012).

Type 2 diabetes is associated with obesity and poor lifestyle, but the cause of insulin resistance is not fully understood. It is thought that there are distinct

populations of patients with different characteristics. Greater understanding of these subgroups would enable a personalised approach to treatment. In 2018, a Scandinavian study of more than 14,000 diabetic patients reported that diabetes should be classified into five distinct clusters (Ahlqvist et al., 2018) (see table 1.1).

Table 1.1: The five distinct characteristics of diabetes

Cluster	*Name of disease*	*Characteristics*
1	Severe autoimmune diabetes	Classically known as the Type 1 diabetes, an autoimmune deficiency leads to β-cells unable to produce insulin. Patients develop the disease at a young age and generally have a healthy weight.
2	Severe-insulin deficient diabetes	Similar age and weight characteristics to cluster 1 and the body struggles to produce insulin, but the diabetes is not caused by an autoimmune disease. Highest risk of retinopathy.
3	Severe-insulin resistant diabetes	Generally overweight patients. The body cannot respond to the insulin being produced. Higher risk of kidney disease than clusters 4 and 5.
4	Mild obesity-related diabetes	Generally, very overweight patients with insulin resistance, who are metabolically closer to normal than the patients in cluster 3.
5	Mild age-related diabetes	They tend to develop insulin resistance later in life and their symptoms are milder than the patients in the other clusters.

A Scandinavian study of more than 14,000 diabetic patients reported that diabetes should be classified into five distinct clusters (Ahlquist et al., 2018).

1.2 Cardiovascular Disease in Type 2 Diabetes Mellitus

Atherothrombosis accounts for the majority of deaths in type 2 diabetic patients (Reusch and Drazin, 2007). It has been reported that diabetic patients have a two- to four-fold increased risk of coronary artery disease (CAD) and peripheral artery disease (Kuusisto and Laakso, 2013). Large-scale studies have shown that type 2 diabetic patients are at equal risk of myocardial infarction as non-diabetic subjects with a prior myocardial infarction (Haffner et al., 1998; Schramm et al., 2008). Supporting the concept that diabetes is a cardiovascular disease equivalent and that diabetic patients should be given primary prevention prior to a myocardial infarction. However, other studies have supported the concept of diabetes as a cardiovascular disease equivalent in sub-groups but not the entire population (Kuusisto and Laakso, 2013). Sub-groups such as age, gender, ethnicity and duration of diabetes can increase the risk of cardiovascular disease. The current National Institute for Health and Care Excellence (NICE) guidelines is to offer antiplatelet therapy (such as aspirin or clopidogrel) for adults with type 2 diabetes as a secondary prevention of cardiovascular disease (NICE, 2018).

The disease accelerates the development of atherosclerosis, resulting in plaque rupture, platelet activation and thrombosis. Growing evidence demonstrates that the mechanisms involved in maintaining the integrity of the vasculature are impaired in type 2 diabetic patients. In healthy vasculature, haemostasis is regulated by a network of endothelial and smooth muscle cells (SMCs). A monolayer of endothelial cells that line the inner arterial wall, release mediators that promote vascular haemostasis, such as nitric oxide (NO) and prostacyclin I_2

(PGI_2), and the SMCs regulate the vascular tone of the arteriole walls (Libby et al., 2011).

In diabetes, this intricate network is perturbed, resulting in the development of atherosclerotic plaques in the inner lining of the arteries, known as atherogenesis (Libby et al., 2011). During atherogenesis, endothelial cells express adhesion molecules to capture leukocytes on their surfaces when subjected to stimuli such as dyslipidaemia, pro-inflammatory molecules and hypertension (Libby et al., 2011). The monocytes move into the innermost layer of the artery, the tunica intima, differentiate into macrophages to engulf biochemically modified low density lipoprotein (LDL) particles and become foam cells, forming the atherosclerotic plaque (Lusis, 2000). Furthermore, SMCs migrate from the tunica media into the tunica intima and produce extracellular matrix proteins, such as collagen and elastin, to form a fibrous cap that covers the plaque (Libby et al., 2011). Rupture of the plaque results in the activation of platelets and coagulation factors to form thrombi that disrupts arterial blood flow, ultimately leading to myocardial infarction or stroke.

The pathogenesis of atherosclerosis in type 2 diabetes is the same as non-diabetic subjects but with the added burden of elevated glucose (Hadi et al., 2005). Driven by hyperglycaemia and oxidative stress, advanced glycation end-products (AGEs) form to alter vessel wall haemostasis and accelerate the progression of atherosclerosis (Hadi and Suwaidi, 2007). Within the vessel wall, collagen-linked AGEs may “trap” plasma proteins and interact with membrane receptors to alter cellular properties. AGEs initiate oxidative reactions that

promote the formation of oxidised-low density lipoprotein (ox-LDL) (Hadi and Suwaidi, 2007). Oxidised lipoproteins and reactive oxygen species (ROS) penetrate the vascular wall, promoting leukocyte adhesion. The attachment of AGEs to endothelium may also quench NO activity to promote platelet activation (Hadi and Suwaidi, 2007).

1.3 The Role of Platelets in Atherothrombosis

The primary role of platelets is to prevent excess blood loss upon vascular injury maintaining haemostasis (Kaplan and Jackson, 2011). This is achieved through the generation of platelet rich clots, which plug the damaged area. Through similar mechanisms, platelets also play a key role thrombosis, the development of pathological clots. In arteries, the most common cause of thrombosis is rupture or erosion of atherosclerotic plaques. The loss of the endothelium and exposure of extracellular matrix proteins results in platelet adhesion to the vascular wall, followed by platelet activation, and aggregation, resulting in atherothrombosis (Kaplan and Jackson, 2011).

In addition to their role in later stages of atherothrombosis, the concept of platelets as key players in the development of atherosclerosis has become increasingly accepted due to accumulating evidence. Under physiological conditions, the intact endothelium prevents platelet adhesion. However, under inflammatory conditions, circulating platelets adhere to the activated endothelial layer via an initial “loose” contact known as platelet rolling (Langer et al., 2017). Activated platelets express adhesive ligands, such as P-selectin and $\alpha_{IIb}\beta_3$ receptors, on their membrane surface, and this leads to aggregate formation and the recruitment of leukocytes. It has been reported that the role of platelets in atherosclerosis might begin in the very early stages of the disease. Using a mouse model for atherosclerosis, Massberg et al. (2002) reported that platelet adhesion to endothelium occurred before the development of the lesion, indicating a proatherogenic platelet phenotype. Using a similar mouse model for atherosclerosis, Burger and Wagner (2003) demonstrated that platelet P-selectin

contributed to lesion development and that P-selectin expression activates monocytes located at the lesion to secrete chemokines that attract or stimulate proliferation of SMCs. Thus, the development of biomarkers for circulating platelets could be important in the early identification of patients most at risk of cardiovascular disease. It also indicates that targeting platelets, in particular the P-selectin pathway, may have potential to reduce atherogenesis.

1.4 Mechanisms of platelet activation and aggregation

Platelets are small cellular fragments that circulate in the bloodstream for approximately ten days before they are cleared by macrophages in the liver and spleen (Daly, 2011).

Platelets are formed from the cytoplasm of their parent cells, megakaryocytes, which reside in the bone marrow. The normal human platelet count is approximately 250-450 x 10^9/L (Giles, 1981). Abnormalities in platelet production can result in two clinically significant disorders. Thrombocytopenia (platelet counts below 150 x 10^9/L) leads to inadequate clot formation and increased bleeding, and thrombocythemia (platelet counts above 600 x 10^9/L) can increase the risk of thrombotic events such as stroke and myocardial infarction (Patel et al., 2005).

Platelets lack a nucleus and hence cannot adapt to different situations by *de novo* protein synthesis (although they do carry over residual messenger RNA (mRNA) from the megakaryocytes) (Clemetson and Clemetson, 2013). Thus, platelets have a number of different receptors that are present in the platelet storage granules and expressed on the surface after activation. Activation of the platelet receptors triggers a series of intracellular events that include the mobilisation of intracellular calcium and phosphorylation of proteins to activate a number of feedback mechanisms (Colwell and Nesto, 2003). These mechanisms bring about morphological and biochemical changes in the activated platelet including shape change, thromboxane A_2 (TXA_2) synthesis and granule secretion.

The main function of platelets is haemostasis, and so the function of the major receptors is to promote thrombus formation either by platelet adhesion to the damaged vessel wall or the activation of other platelets to the site of injury. The steps in platelet thrombus formation are sometimes described as initiation, extension and stabilisation (Brass et al., 2013). Initiation occurs when the platelets tether to exposed von Willebrand factor (vWF)/collagen complexes on a monolayer of endothelial cells, enabling platelet adhesion to the damaged vessel wall. Extension occurs when additional platelets adhere to the endothelium and become activated. Thrombin, adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2) released by platelets play an important role in this step. Finally, stabilisation refers to the subsequent steps, particularly outside-in signalling, that help to stabilise the thrombus so that it cannot disaggregate (Brass et al., 2013). These three steps will be discussed in more detail below.

Under high shear stress (typically at sites of atherosclerosis where stenosis of the vessel lumen occurs), platelets are tethered to exposed sub-endothelial collagen fibres by binding of immobilised von Willebrand factor (vWF) to the Glycoprotein (GP) Ibα in the platelet membrane GPIb-IX-V complex (Ruggeri and Mendolicchio, 2007). Circulating erythrocytes facilitate platelet adhesion to collagen by pushing platelets closer to the sub-endothelium allowing GPIbα to be snared by the vWF A1 domain (Brass et al., 2013). This tethering facilitates binding of collagen to the low-affinity GPVI receptor on platelets and the initiation of intracellular signalling that leads to inside-out activation of the integrin receptors $\alpha2\beta1$ and $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb/IIIa), as well as further clustering of GPVI (Nieswandt et al., 2001). These signals mediate stable platelet

adhesion and spreading through binding of $\alpha 2\beta 1$ and $\alpha_{IIb}\beta_3$ to collagen and vWF, respectively. Platelets have a low-level constitutively active $\alpha 2\beta 1$ that can bind to collagen at lower shear rates. However, GPVI plays the predominant role in mediating platelet adhesion at high shear rates (Pollitt et al., 2013).

Once platelets adhere to the sub-endothelial collagen, the primary drivers for platelet activation include the signalling events that occur downstream of collagen (GPVI), thrombin (protease-activated receptor [PAR] 1 and PAR4), ADP ($P2Y_1$ and $P2Y_{12}$) and TXA_2 (TP) (Brass et al., 2013). Clustering of GPVI leads to an intracellular signalling cascade involving phosphorylation in the Fc receptor γ chain ($Fc R\gamma$) cytoplasmic domain, followed by activation of Syk, and phosphorylation and activation of phospholipase $C\gamma_2$ ($PLC\gamma_2$) (Kasirer-Friede et al., 2004). This hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and Diacylglycerol (DAG) (Bye et al., 2016). IP_3 opens up calcium channels in the platelet dense tubular system and DAG activates the PKC isoforms expressed in platelets to allow for serine/threonine phosphorylation events needed for platelet activation (Brass et al., 2013). One consequence of $PLC\gamma_2$ activation is the production and release of the secondary agonists TXA_2 and ADP, which bind to circulating platelets to further increase thrombus formation (Brass et al., 2013).

TXA_2 stimulates vasoconstriction, granule secretion, and initiates secondary aggregation (Gawaz, 2001), however, it does not have a direct effect on the formation of the platelet inhibitory molecule, cAMP (Brass et al., 2013). Synthesis of TXA_2 occurs when calcium is released from the intracellular storage pools and

activates phospholipase A_2. This enzyme hydrolyses the membrane phospholipids to arachidonic acid, which is converted to TXA_2 via the aspirin-sensitive cyclooxygenase (COX)-1 pathway (Angiolillo, 2009). TXA_2 can diffuse across the membrane and activate other platelets (Brass et al., 2013).

Calcium activation and serine/threonine phosphorylation results in the secretion of ADP from the dense granules and binding to the purinergenic receptors, $P2Y_1$ and $P2Y_{12}$. *In vitro* studies have shown that ADP causes TXA_2 synthesis, shape change, aggregation and secretion (Brass et al., 2013). It also inhibits cyclic adenosine monophosphate (cAMP) formation, which, in turn, diminishes the platelet inhibitory response. When $P2Y_1$ is blocked or deleted, there is an impaired response to ADP (e.g. absent increase in calcium and shape change). Nevertheless, ADP is still able to inhibit cAMP formation (Léon et al., 1999). However, when $P2Y_{12}$ is deleted in mice, there is a greatly diminished aggregation and the mice also fail to inhibit cAMP formation in response to ADP (Foster et al., 2001). Drugs that block $P2Y_{12}$, such as clopidogrel and ticlopidine, have proved to be effective antiplatelet agents (Savi and Herbert, 2005).

Exposure of phosphatidylserine on the surface of activated platelets enables the assembly of the prothrombin complex and generation of thrombin (Bye et al., 2016). Thrombin is a strong agonist and able to activate platelets at concentrations as low as 0.1 nM, making it the most potent agonist (Brass et al., 2013). It appears to cause phosphoinositide hydrolysis and the increase of cytosolic calcium more rapidly than other agonists. Human platelets express PAR1 and PAR4, and activation of either receptor by thrombin triggers rapid

platelet activation and aggregation. Receptor activation occurs when thrombin cleaves the extended N-terminus of either PAR1 or PAR4 that serves as a tethering ligand, binding intramolecularly to the body of the receptor to effect transmembrane signalling (De Candia, 2012). The $P2Y_{12}$ and PAR-1 pathways are known to cross-react in mediating platelet activation (Angiolillo et al., 2010). Unlike ADP, which directly activates both Gαi (via $P2Y_{12}$) and Gαq (via $P2Y_1$) signalling, thrombin cannot directly activate both signals and requires the secondary Gαi signal from $P2Y_{12}$ and secreted ADP (thrombin's secondary response) to complement the Gαq signals from PAR-1 and PAR-4.

The stabilisation stage is the final step whereby cohesive contacts between platelets enable the thrombus to become stable. The receptor-bound ligands (fibrinogen, vWF or fibrin) act as a bridge between two $\alpha_{IIb}\beta_3$ molecules on adjacent platelets to form the stable thrombus (Brass et al., 2013). $\alpha_{IIb}\beta_3$ is the only receptor uniquely expressed on platelets, with 50,000 to 80,000 copies per platelet (Clemetson and Clemetson, 2013). It is activated by inside-out signalling via the platelet signalling events initiated by collagen, thrombin, ADP and TXA_2 (Bledzka et al., 2013), which gives rise to conformational changes within $\alpha_{IIb}\beta_3$. The common event induced by these agonists is activation of a PLC isoform, elevation of cytosolic calcium and activation of PKC. Although each of the stimuli is capable of initiating inside-out signalling, it is likely that they act co-operatively (Bledzka et al., 2013). See figure 1.1 for an overview of the major signalling pathways of platelet activation.

Although the primary role of platelets is to maintain haemostasis, they also contribute to inflammation through platelet-leukocyte interactions. P-selectin is a cell adhesion molecule involved in this interaction and it is located in the α-granules of resting platelets (Blann et al., 2003). It is also located in the Weibel–Palade bodies of endothelial cells. Upon activation, P-selectin translocates to the platelet surface, whereby it mediates the interaction with leukocytes that express P-selectin glycoprotein ligand-1 (PSGL-1). Here it facilitates the rolling of platelets and neutrophils on activated endothelial cells, and the interaction of platelets with monocytes and neutrophils (Blann et al., 2003). There are approximately 10,000 P-selectin molecules on the surface of an activated platelet (Blann et al., 2003).

Besides leukocyte recruitment and platelet-leukocyte interactions, P-selectin also has a role in platelet aggregation. Merten and Thiagarajan (2000) demonstrated that P-selectin was maximally expressed ten minutes after maximal $\alpha_{IIb}\beta_3$ activation, and that platelet aggregate size and stability depends on P-selectin. Michelson et al. (1996) showed *in vivo* that P-selectin is rapidly cleaved from degranulated platelets and released into the plasma pool as soluble P-selectin (sP-selectin), but the degranulated platelets continue to circulate and have the ability to form aggregates.

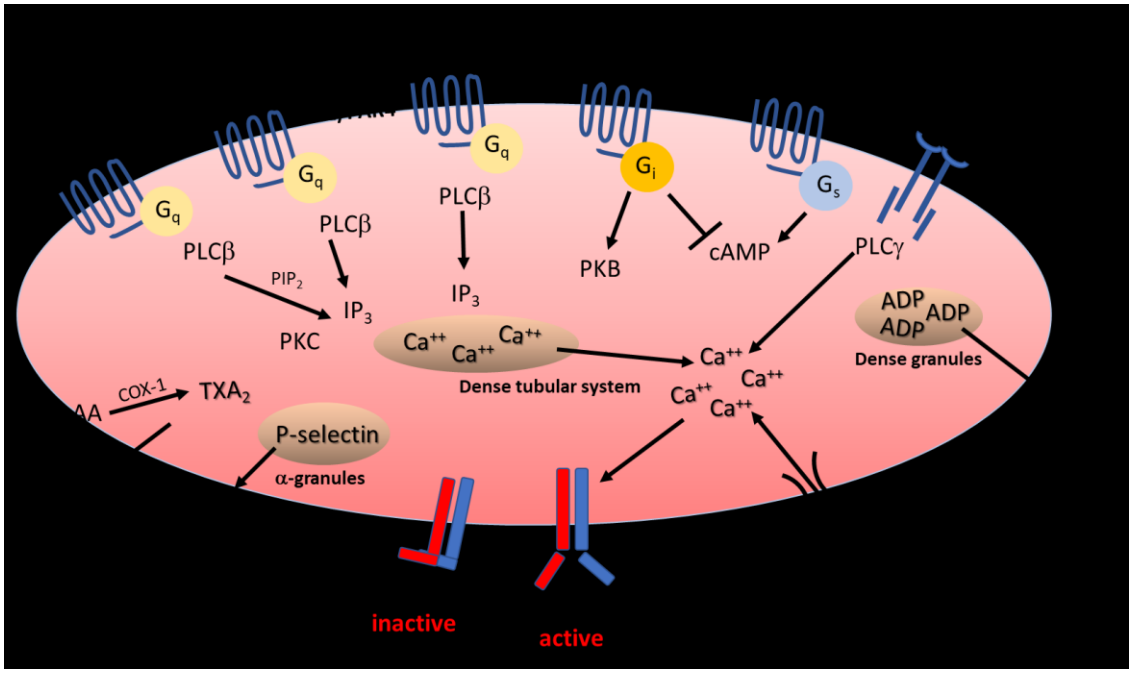

Figure 1.1: An overview of the major signalling pathways of platelet activation

Once platelets adhere to damaged endothelium, the primary drivers for platelet activation include the signalling events that occur downstream of collagen (GPVI), thrombin (protease-activated receptor [PAR] 1 and PAR4), ADP ($P2Y_1$ and $P2Y_{12}$), and TXA_2 (TP). In general terms, agonists initiate activation of a phospholipase C (PLC) isoform, which hydrolyses phosphatidylinositol-4,5-biphosphate (PIP_2) to produce the second messenger, inositol-1,4,5-triphosphate (IP_3), needed to raise the calcium (Ca^{++}) levels. This results in a conformational change in $\alpha_{IIb}\beta_3$ which binds fibrinogen and is the final step in platelet aggregation. P-selectin translocates to the membrane to recruit leukocytes. Other abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; COX-1, cyclooxygenase-1; GP, glycoprotein; PGI_2, prostacyclin; PKB, protein kinase B; PKC, protein kinase C. Diagram adapted from Brass et al. (2013).

1.5 Mechanisms of Platelet Inhibition

Within the circulation, each platelet is balanced by approximately fifty endothelial cells (Aird, 2005). The endothelium modulates vascular tone and platelet inhibition by co-releasing the endothelium-derived vasodilator factors NO and PGI_2 (Nucci et al.,1988). Endothelial cells constitutively express endothelial nitric oxide synthasGPe (eNOS) that, upon calcium binding, generates NO using L-arginine together with other co-factors (Dhananjayan et al., 2016). NO diffuses freely into platelets where it binds to the soluble intracellular receptor, guanylyl cyclase, leading to an increase in cyclic guanosine monophosphate (cGMP) levels and reduced intracellular calcium (Jensen et al., 2004). The decrease in calcium levels inhibits the conformational change of $\alpha_{IIb}\beta_3$ into its active form, and thus decreases platelet dissociation with fibrinogen (Beaulieu and Freedman, 2013). NO is important not only for inhibiting aggregation and adhesion, but also for modulating platelet function itself. Platelet-derived NO is released from both resting and activated platelets to regulate platelet reactivity (Beaulieu and Freedman, 2013).

PGI_2 is a potent vasodilator and antithrombotic, and it also inhibits the growth of SMCs (Beaulieu and Freedman, 2013). PGI_2 binds to platelet IP receptors to mediate activation of adenylyl cyclase, which leads to an increase in cAMP and reduced intracellular calcium (Mitchell et al., 2008). The biosynthesis of PGI_2 is part of the arachidonic pathway, the same pathway that produces TXA_2. It is synthesised in endothelial cells and SMCs, but not platelets (Beaulieu and Freedman, 2013).

In addition to PGI_2 and NO, ecto-nucleotidases play an important inhibitory role in modulating platelet reactivity. They rapidly hydrolyse activatory ATP and ADP to AMP and adenosine, which negatively regulates platelet activity (Lunkes et al., 2008). These include the nucleoside triphosphate diphosphohydrolase (NTPDase), CD39, and the 5'-nucleotidase, CD73, located in the platelet membrane.

There is an interplay between the endothelial-derived anti-aggregatory molecules and the platelet-derived pro-aggregatory molecules. For example, the activation of the $P2Y_{12}$ receptor by ADP causes inhibition of sAC and this reduces the ability for PGI_2 (acting through IP receptors) to elevate cAMP, (Cattaneo and Lecchi, 2007). Therefore, inhibition of the $P2Y_{12}$ receptor potentiates the inhibitory effects of PGI_2. In addition to this, Kirby et al. (2013) reported that the ability of NO to inhibit platelet aggregation was largely dependent on the inhibition of the $P2Y_{12}$ receptor. This is despite the fact that NO acts through a different platelet pathway to PGI_2, by activating sGC and causing increased cGMP. Another study has demonstrated that ADP-induced secondary aggregation is dependent on the inhibition of ecto-nucleotidases (Jones et al., 2011).

1.6 Platelet Dysfunction in Type 2 Diabetes

The importance of platelets in atherothrombosis has led to the development of antiplatelet drugs, such as aspirin and clopidogrel, to reduce the risk of cardiovascular events. Although most antiplatelet drugs irreversibly inactivate platelet function (Angiolillo, 2009), large-scale studies indicate that type 2 diabetic patients are less responsive to the medication than non-diabetic patients (Mehta et al., 2000; Ogawa et al., 2009; Belch et al., 2008). Numerous studies have reported that type 2 diabetic patients exhibit enhanced platelet activation and aggregation (Vinik et al., 2001), and reduced sensitivity to antiplatelet drugs (Angiolillo et al., 2005; Schuette et al., 2015). They also exhibit increased expression of membrane receptors such as $P2Y_{12}$ (Hu et al., 2017). There is a large body of data suggesting potential mechanisms responsible for the enhanced platelet reactivity including elevated release of TXA_2 (Halushka et al. 1981; Davi et al., 1982), reduced sensitivity to the platelet inhibitors NO and PGI_2 (Nolan et al., 1994; Akai et al., 1983), and increased expression of the activation-dependent adhesion molecules $\alpha_{IIb}\beta_3$ and P-selectin (Angiolillo, 2005), however the mechanisms are not fully understood.

Type 2 diabetes is multifactorial and associated with biochemical factors such as hyperglycaemia, insulin resistance, hyperlipidaemia, chronic inflammation and oxidative stress (Kakouros et al., 2011). Although there has been a large amount of research focusing on platelet dysfunction in diabetes, a direct connection between platelet hyperreactivity and diabetic characteristics has yet to be firmly established. Identifying the specific biochemical alterations in diabetic patients that correlate with platelet reactivity, could be a useful tool for identifying patients

at increased risk of thrombosis and those who would therefore benefit from antithrombotic therapy. Understanding the mechanisms responsible for altered platelet function and antiplatelet resistance will also facilitate the development of novel, more efficacious antiplatelet treatment for these high-risk patients.

The following sub-chapters discuss the literature that has focused on the association between platelet hyperreactivity and biochemical risk factors (including hyperglycaemia, insulin resistance, hyperlipidaemia and inflammation), and highlights the conflicting views that emphasizes the need for further research in this field.

1.7 Hyperglycaemia and Platelet Dysfunction

Hyperglycaemia is a hallmark of diabetes, and large-scale studies have shown a strong relationship between hyperglycaemia and cardiovascular disease in type 2 diabetic patients. A recent observational study of more than 32,700 type 2 diabetic patients reported that an HbA1c result over 64 mmol/mol increases the risk of macrovascular complications (van Wijingaarden et al., 2017). An observational study of more than 3,600 type 2 diabetic patients demonstrated that a 1% reduction in HbA1c was associated with a risk reduction of 14% for myocardial infarction and 37% for microvascular complications (Stratton et al., 2000). A meta-analysis of published data from twenty studies demonstrated that a 4.2 to 9 mmol/L rise in FBG increased the relative risk of cardiovascular events from 1 to 2.2 (Coutinho et al., 1999). In addition, a meta-analysis of four large studies that measured cardiovascular outcomes from intensive glycaemic control in type 2 diabetic patients, showed a 15% reduced risk of myocardial infarction and 9% reduction in major cardiovascular events (Turnbull et al., 2009).

Although intensive glycaemic control appears to reduce the risk of cardiovascular events, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study reported a previously unrecognised harm of intensive glucose lowering in type diabetic patients with a target <48 mmol/mol (6.5%) HbA1c (Gerstein et al., 2008). The ACCORD study was terminated due to increased mortality in the intensively treated group, creating an ongoing debate on the potential link between hypoglycaemia and increased rate of cardiovascular disease in type 2 diabetic patients. Consequently, the optimal glucose level remains unknown (Ferreiro et al., 2010).

The increased risk of cardiovascular risk can exist for many years before the onset of hyperglycaemia (Schofield et al., 2016). During this period, patients show symptoms of metabolic syndrome, which include obesity, insulin resistance, hypertension and dyslipidaemia. The United Kingdom Prospective Diabetes Study (UKPDS) observed that one in six newly diagnosed type 2 diabetic patients had evidence of previous silent myocardial infarction (Davis et al., 2013). Indicating that other risk factors may lead to the early development of CAD.

Chronic hyperglycaemia is associated with the accumulation of endogenous AGEs, formed by the non-enzymatic reaction (Maillard reaction) between a carbonyl group of reducing sugars and the free amino groups from proteins, phospholipids and nucleic acids (Chilelli et al., 2013). Binding of AGEs to the receptor for advanced glycation end-products (RAGE) enhances oxidative stress, inflammation and apoptosis (Stirban et al., 2014). A key mediator of RAGE signalling is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa\beta$), which translocates to the nucleus where it increases transcription of proinflammatory and prothrombotic genes (Li and Schmidt, 1997).

Western diet is related to the accumulation of circulating AGEs in type 2 diabetes, and this was associated with an increase in markers for inflammation and endothelial dysfunction (Negrean et al., 2007). Incubation of platelets with AGEs derived from Coca-Cola™ increased the expression of platelet activation markers up to 7.1-fold, whilst increasing expression of the receptor for AGEs, RAGE (Gawlowski et al., 2009). Though no evidence of an AGE-RAGE interaction was confirmed. A later study showed that platelets express RAGE on their surface

(Zhu et al., 2012). However, treatment of platelets with a RAGE-blocking antibody did not affect specific binding of AGE to the platelet surface. Given that platelets do not have nuclei, and that RAGE induces signalling through the transcription factor NF-κβ, it is unclear what role RAGE plays in platelets. Of note, Zhu et al. (2012) did identify AGEs bound to platelet CD36 a scavenger receptor that recognises modified lipoproteins.

1.7.1 Platelet Indices and Hyperglycaemia

Platelet indices are a group of platelet parameters measured using an automated haematologic analyser and includes platelet count, mean platelet volume (MPV), platelet distribution width (PDW) and platelet-larger cell ratio (P-LCR) (Budak et al., 2016). MPV quantifies the average size of the platelet population. It is used as a biomarker for platelet activation because the larger platelets tend to be younger and more reactive (Grove et al., 2011). MPV is associated with cytokines (e.g. thrombopoietin, interleukin (IL)-6 and IL-3) that regulate megakaryocyte ploidy and platelet number, and result in the production of larger platelets (Budak et al., 2016). When platelet production is decreased, younger platelets become bigger and more active, and MPV levels increase. PDW is an indicator of volume variability in platelet size and reflects the heterogeneity in platelet morphology, and P-LCR is an indicator of circulating larger platelets (>12 fL), presented as a percentage (Budak et al., 2016).

Several studies have investigated the relationship between MPV and hyperglycaemia *ex vivo*. The majority of them found a positive correlation between high MPV and HbA1c in type 2 diabetic patients (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder and Eker, 2014; Ulutas et al., 2014). However, data from Hekimsoy et al. (2004) found no relationship between HbA1c and MPV (see table 1.2).

Data from studies investigating the relationship between MPV and FBG is more inconsistent. Some report a strong relationship between FGB and MPV in type 2

diabetic patients (Kodiatte et al., 2012; Ozder and Eker, 2014; Ulutas et al., 2014) and other studies found no relationship (Demirtunc et al., 2009; Hekimsoy et al., 2004) (see table 1.3). In addition, Shimodaira et al. (2013) found a relationship between MPV and FBG in prediabetic subjects. The contradictory data from *ex vivo* studies questions the use of MPV as a marker for cardiovascular risk in patients with hyperglycaemia.

Although the data is contradictory for correlations between MPV and hyperglycaemia, several studies have calculated a significant difference between type 2 diabetic patients and non-diabetic subjects (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder and Eker, 2014; Ulutas et al., 2014). These include studies that found no relationship between MPV and HbA1c (Hekimsoy et al. (2004) and Pananas et al. (2004) (see table 1.4), indicating that additional factors are involved.

Table 1.2: Relationship between MPV and HbA1c in type 2 diabetic patients

Source of data	*MPV (fL) (mean ± SD)*	*HbA1c (%) (mean ± SD)*	*R value*	*P value*	*n*
Kodiatte et al (2012)	8.29 ± 0.735	9.13 ± 2.5	0.29	<0.001	300
Demirtunc et al. (2009)	8.7 ± 0.8	8.4 ± 1.2‡	0.394	0.001	35
Ozder and Eker (2014)	10.66 ± 0.94	8.80 ± 1.62	0.357	0.000	201
Ulutas et al. (2014)	8.3 ± 1.3	9.6 ± 2.3‡	0.393	<0.001	32
Hekimsoy et al. (2004)	10.62 ± 1.71	7.49 ± 1.5	-0.033	0.74 NS	145

‡Diabetic cohort with an HbA1c >7% was used. NS: data is not significant.

Table 1.3: Relationship between MPV and FBG in type 2 diabetic patients

Source of data	*MPV (fL) (mean ± SD)*	*FBG (mmol/L) (mean ± SD)*	*R value*	*P value*	*n*
Kodiatte et al (2012)	8.29 ± 0.735	8.35 ± 3.98	0.269	<0.001	300
Demirtunc et al. (2009)	8.7 ± 0.8	12.21 ± 4.2‡	Not reported	NS	35
Ozder and Eker (2014)	10.66 ± 0.94	12.25 ± 3.50	0.306	0.000	201
Ulutas et al. (2014)	8.3 ± 1.3	13.68 ± 4.57‡	0.41	<0.001	32
Hekimsoy et al. (2004)	10.62 ± 1.71	9.38 ± 2.89	0.099	0.24 NS	145

*Diabetic cohort with an HbA1c >7% was used. NS: data is not significant.

Table 1.4: Mean MPV in type 2 diabetic subjects and non-diabetic subjects from six separate literature sources

Source of data	*MPV (fL) in non-diabetics (mean ± SD)*	*N*	*MPV (fL) in type 2 diabetic patients (mean ± SD)*	*n*	*P value*
Kodiatte et al (2012)	7.47 ± 0.726	300	8.29 ± 0.735	300	<0.001
Demirtunc et al. (2009)	8.2 ± 0.7	40	8.7 ± 0.8‡	35	0.002
Ozder and Eker (2014)	10.04 ± 1.01	201	10.66 ± 0.94	201	0.000
Ulutas et al. (2014)	7.1 ± 1.0	40	8.3 ± 1.3‡	32	<0.001
Hekimsoy et al. (2004)	9.15 ± 0.86	100	10.62 ± 1.71	145	0.00
Pananas et al. (2004)	7.1 ± 1.2	151	14.2 ± 2.2	131	0.01

‡Diabetic cohort with an HbA1c >7% was used. This cohort is considered to have uncontrolled diabetes. All studies used the unpaired t-test except those marked with a ‡ that used the one-way ANOVA.

In contrast to the data shown in table 1.4, Shlomai et al. (2015) calculated an MPV of 9.2 ± 1.3 fL for both diabetic and non-diabetic patients (n=82 and n=86, respectively). This is an important finding because this was a well-designed study with stringent exclusion criteria. The two groups were matched for age, body mass index (BMI), hypertension, hyperlipidaemia, smoking, medication, co-morbidities and renal function. All diabetic subjects had well controlled glycaemia (the mean HbA1c was 49.7 mmol/mol) and no prior ischemic events. Conversely, the limitation of the study was that the diabetic patients had good glycaemic control so any possible effects caused by chronic hyperglycaemia may not have been observed.

It is interesting to note that there was no correlation for MPV vs. duration of diabetes (Kodiatte et al., 2012; Demirtunc et al. 2009; Hekimsoy et al., 2004) and no significant correlation was shown for MPV vs. vascular complications (Demirtunc et al., 2009; Kodiatte et al., 2012). Demirtunc et al. (2009) hypothesised that this was caused by the rapid consumption of activated platelets in diabetic patients with complications. This is supported by literature that investigated MPV in other diseases. For example, Bilen et al (2015) found a decrease in MPV in non-diabetic patients with renal failure. In patients with high-grade inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus, MPV can be low during the active stage of the disease, suggesting that the larger, more active platelets are consumed at the sites of inflammation (Gasparyan et al., 2011).

Although the majority of the literature indicates that MPV is increased in type 2 diabetic patients compared to those without diabetes, it is important to note that there was no definitive explanation for this outcome. For example, *ex vivo* studies cannot demonstrate whether the high MPV is simply caused by osmotic swelling due to raised levels of glucose metabolites. Alternatively, the increased MPV in type 2 diabetes could be an indication of a direct increase in thrombopoiesis or enhanced platelet turnover caused by their propensity to atherosclerosis. Therefore, if an atherosclerotic plaque ruptures, platelets are required to plug the damaged site and so the bone marrow produces more platelets (Kodiatte et al. 2012).

1.7.2 Platelet Production and Hyperglycaemia

Larger platelets are metabolically and enzymatically more active, and have greater prothrombotic potential. Furthermore, they are often immature platelets that contain residual messenger ribonucleic acid (mRNA) and a greater number of dense granules (Mijovic et al., 2015). They are called reticulated platelets due to the staining pattern of the cytoplasmic mRNA distribution. A pilot study using type 2 diabetic patients (n=30) and non-diabetic controls (n=49), showed that the percentage of reticulated platelets in type 2 diabetic patients was significantly higher than non-diabetic subjects (Mijovic et al., 2015). Additionally, Cesari et al. (2013) identified a higher percentage of reticulated platelets in those that died within a year of being diagnosed with acute coronary syndrome (ACS).

Aspirin is the most widely used drug for the prevention of cardiovascular disease. It irreversibly blocks the COX-1 enzyme, inhibiting the platelets for their entire life, yet diabetic patients have a lower response to aspirin than non-diabetic patients (Angiolillo, 2009). Insufficient platelet inhibition could be explained by increased thrombopoiesis, yielding an increased subpopulation of younger, more reactive platelets (Grove at al., 2011). Newly produced platelets are more capable of producing membrane and secretory proteins because of the considerable amount of megakaryocyte-derived mRNA. This could give rise to more reactive platelets that are less able to respond to antiplatelet therapy or a subpopulation of newly formed platelets that have not been exposed to appropriate doses of aspirin.

The rate of thrombopoiesis can be quantified using flow cytometry with fluorescent staining of RNA. It can be measured as absolute immature platelet count (IPC), or immature platelet fraction (IPF) as a ratio of immature platelets to total platelet count and is given as a percentage (Grove at al., 2011). In a study looking at both IPC and IPF in a cohort with stable CAD, Grove et al. (2011) identified a 17-21% rise in immature platelets in the type 2 diabetic sub-group. Interestingly, they noted that IPC significantly correlated with platelet aggregation and sP-selectin in the CAD population, whereas IPF did not. Furthermore, IPC was not dependent on the total platelet count.

Neergaard-Petersen et al. (2015) also looked at platelet turnover in a CAD population but in relation to hyperglycaemia. They separated the groups into non-diabetic, prediabetic and type 2 diabetes. This was a beneficial study because the prediabetic cohort had moderately elevated glucose levels but were not taking antidiabetic treatment. All participants were treated with low dose aspirin. A number of important findings were reported:

1. CAD patients with diabetes had higher platelet aggregation, sP-selectin levels and platelet turnover compared with non-diabetic CAD patients.
2. Platelet count and MPV did not differ between the non-diabetic and diabetic CAD patients.
3. Prediabetic patients had increased aggregation, platelet count and platelet turnover compared with the non-diabetic cohort. However, they did not have increased sP-selectin levels.

4. HbA1c correlated significantly with platelet aggregation, sP-selectin, IPC and platelet count in the whole CAD population; but did not correlate with MPV and IPF.
5. Most importantly, HbA1c did not correlate with platelet aggregation, sP-selectin, platelet turnover, platelet count and MPV in the diabetic cohort alone. However, it did correlate with all parameters in the non-diabetic CAD cohort.

Neergaard-Petersen et al. (2015) hypothesised it is the antidiabetic treatment, such as Metformin and insulin, that partly explains the lack of correlation between HbA1c and platelet reactivity in the diabetic patients with CAD. Literature into the effect of Metformin on platelets is limited, although MPV was shown to significantly reduce baseline MPV in type 2 diabetic patients after six months of treatment (Dolasık et al., 2013). Additionally, studies related to the effect of insulin on platelet reactivity is contradictory (Trovati et al., 1988; Yngen et al, 2001; Rauchfuss et al., 2008). It is also interesting to note that the platelet count was elevated in the prediabetic cohort but not the diabetic cohort.

More recently, Fidler et al. (2017) showed that glucose metabolism is required for megakaryocyte-mediated platelet production. Glucose transporter (GLUT) 1 and GLUT3 double knock-out mice developed thrombocytopenia through decreased platelet production and increased platelet clearance. Although megakaryocyte density in the bone marrow was unchanged, the inability of the megakaryocytes to take up glucose almost completely abolished their ability to produce platelets.

1.7.3 Platelet Activation and Hyperglycaemia

Many studies have investigated hyperglycaemia as a risk factor for increased platelet activation and aggregation, though studies have shown conflicting data. Singer et al. (2014) measured platelet aggregation and sP-selectin in thirty diabetic patients before and after glycaemic control over a four-month period. The mean HbA1c at baseline was 9.4% (79.2 mmol/mol) and decreased to 8.1% (65 mmol/mol). Singer et al. (2014) did not find a significant difference in platelet aggregation stimulated with arachidonic acid and ADP using light transmission aggregometry (LTA). Neither did they find a significant difference in P-selectin.

Following ADP activation, Shlomai et al. (2015) looked at markers for P-selectin expression and $\alpha_{IIb}\beta_3$ activation in diabetic and non-diabetic participants, closely matched for a number of clinical characteristics. Using flow cytometry, they found no significant difference between the percentage of cells positive for P-selectin expression and $\alpha_{IIb}\beta_3$ activation. However, the diabetic cohort had good glycaemic control and this could be the reason for the lack of difference between the two groups.

A small study using type 2 diabetic and non-diabetic participants (n=14 and n=7, respectively) identified a significant increase in P-selectin expression and $\alpha_{IIb}\beta_3$ activation in response to ADP in both cohorts, after whole blood was incubated with 25 mmol/L glucose for one hour (Keating et al., 2003). They concluded that acute hyperglycaemia has a direct effect on platelet activity as a result of changes in osmolarity. Sudic et al. (2006) showed that 30 mmol/L glucose increased ADP-

stimulated P-selectin expression but not $\alpha_{IIb}\beta_3$ activation in whole blood. However, whole blood stimulated with thrombin receptor-activating peptide (TRAP) showed an increase in both P-selectin expression and $\alpha_{IIb}\beta_3$ activation with 30 mmol/L glucose (Sudic et al., 2006).

Tang et al. (2011) demonstrated that 25 mmol/L glucose increased platelet aggregation and P-selectin expression in platelet rich plasma (PRP) with collagen but not with ADP. De la Cruz et al. (2004) demonstrated a dose-dependent increase in platelet aggregation with both collagen and ADP stimulation in whole blood, using lesser physiological glucose concentrations (5-16 mmol/L). Additionally, acute hyperglycaemia significantly elevated P-selectin expression in platelets from type 2 diabetic males compared to healthy controls following an oral glucose tolerance test (Yngen et al., 2001).

Clearly there is conflicting data to show whether acute hyperglycaemia has an effect on platelet activation and aggregation. Notably with ADP and collagen-stimulated platelet reactivity using healthy blood. There may be lots of variables between the studies (e.g. agonist concentration, glucose concentration, flow cytometry thresholds) which are affecting the outcomes of the studies. Further research is required to investigate the effect of glucose on platelets.

1.7.4 Platelet inhibition and hyperglycaemia

Type 2 diabetes is associated with endothelial dysfunction, defined as an imbalance in the production of vasodilator factors that results in the predisposition towards prothrombotic effects (Dhananjayan et al., 2016).

Endothelial dysfunction is commonly associated with the impairment of reduced NO bioavailability, resulting from altered eNOS expression (Schäfer and Bauersachs, 2008). Endothelial cells exposed to high glucose generate mitochondrial ROS and this leads to oxidisation of the eNOS co-factor, tetrahydrobiopterin, in a process known as “eNOS uncoupling” (Edelstein et al., 2000).

Loss of NO bioavailability has been suggested as a major contributor to platelet hyperreactivity in diabetes. Studies using mice with streptozoticin-induced diabetes have demonstrated that enhanced platelet reactivity (observed as reduced vasodilator-stimulated phosphoprotein (VASP) phosphorylation and increased surface expression of P-selectin, CD40L and activated $\alpha_{IIb}\beta_3$) is abolished when eNOS-tetrahydrobiopterin dissociation is prevented in the vascular endothelium (Shäfer et al., 2004).

Some *in vitro* studies have examined the effect of hyperglycaemia on the inhibitory mechanism of NO. Sudic et al. (2006) reported no influence of a NO synthase inhibitor on platelet activation under high glucose conditions, indicating that endogenous platelet NO is not impaired by high glucose. Aspirin has been reported to increase the synthesis of NO in platelets in the absence of endothelial

cells (Madajka et al., 2003), specifically through the acetylation of the platelet NO synthase type 3 (NOS-3) (O'Kane at al., 2009). Russo et al. (2012) found that high glucose had no effect on the NO/cGMP pathway per se, although it did impair the ability of aspirin to activate the NO pathway, potentially through the interference of NOS-3 acetylation by ROS.

Studies have also found a relationship between hyperglycaemia and ecto-nucleotidase activity. Lunkes et al. (2003) found that ecto-nucleotidase activity was increased in diabetic patients, even though they have a predisposition to thrombus formation. To further investigate this paradox, Lunkes et al. (2008) evaluated the effect of different glucose levels on the hydrolysis of adenine nucleotides, and measured CD39 expression in type 2 diabetic and hypertensive patients. They reported a dose-dependent increase in ecto-nucleotidase activity with increasing glucose (5-30 mmol/L). They also observed a significant increase in CD39 platelet membrane expression of diabetic and hypertensive patients. Taking these findings into consideration, it is possible that the rise in ecto-nucleotidadse activity in high glucose is a compensatory mechanism to circumvent platelet hyperreactivity, and that this mechanism of thrombo-regulation is not working efficiently in diabetic and hypertensive patients.

1.8 Insulin Resistance and Platelet Dysfunction

Individuals can exhibit insulin resistance and subsequent hyperinsulinemia for more than twenty years before developing type 2 diabetes, and apoptosis of pancreatic β-cells leads to a relative deficiency of insulin (Schneider, 2009a). A human platelet has about 570 insulin receptors (Hajek et al., 1979), therefore, insulin should be a prominent target of interest for research into platelet hyperreactivity in diabetes. Research into the effect of insulin on platelet function is, however, less than consistent. Insulin has been reported to inhibit (Trovati et al., 1988) increase (Yngen et al. 2001) and not have any effect on platelet function (Rauchfuss et al., 2008).

Early research into the effect of insulin on platelet function was carried out by Trovati et al. (1988). They incubated PRP with insulin from fasting healthy subjects and observed a significant reduction in platelet activity to a number of aggregating agents including ADP and collagen. They also noticed that insulin had both dose and time-dependent effects on the platelets. They later demonstrated that insulin can inhibit platelet activation by increasing intraplatelet concentrations of the inhibitory second messengers, cGMP and cAMP (Trovati et al., 1994, Trovati et al., 1997). These second messengers act predominantly to reduce cytosolic calcium by different mechanisms within platelets (Trovati and Anfossi, 1998). The team also showed that insulin-induced cGMP and cAMP increase is mediated by the platelet inhibitor, NO (Trovati et al., 1997).

Contrary to this, evidence suggests that insulin can enhance platelet activation. Yngen et al. (2001) performed whole blood flow cytometry and LTA using blood

from healthy subjects. Both the whole blood and PRP was incubated with varying concentrations of insulin (1-10,000 μU/ml) and induced with 0.3 μM ADP. When using flow cytometry, a small, but significant, enhancing effect was shown with physiological concentrations of insulin (<100 μU/ml). The greater enhancing effects were shown for $\alpha_{IIb}\beta_3$ activation at supraphysiological concentrations of insulin (>100 μU/ml). P-selectin was less affected by insulin. It is interesting to note that with a higher concentration of ADP (1 μM), insulin only had a slight enhancing effect on $\alpha_{IIb}\beta_3$ activation and no effect on P-selectin. They observed no change with the addition of glucose or calcium. This both challenges and agrees with the data obtained by Anfossi et al. (1996) who showed that ADP-induced platelet aggregation decreased with physiological and slight supraphysiological concentrations of insulin (35-280 μU/ml) and increased with highly supraphysiological concentrations (>3,500-28,000 μU/ml). To support this data, they demonstrated that intraplatelet cGMP levels increased with physiological insulin concentrations and decreased with highly supraphysiological insulin concentrations. A very early publication has provided a possible explanation for insulin's ability to enhance platelet aggregation. Lopez-Aparicio et al. (1992) revealed that insulin can induce phosphorylation and activation of the cGMP-inhibited cAMP phosphodiesterase (PDE) 3 in human platelets, leading to a decrease in cAMP and subsequent increase in platelet aggregation.

It has been suggested that one of the reasons for the varying platelet responses to insulin could be different sources of insulin and solvents. Rauchfuss et al. (2008) tested a commercially available insulin with the solvent and the solvent

alone. They showed that the pure solvent activated platelets similarly to the insulin/solvent mixture. Subsequently, the research team used an insulin polypeptide dissolved in pure water for further experimentation. Interestingly, they demonstrated that the pure form of insulin failed to alter thrombin-stimulated $\alpha_{IIb}\beta_3$ activation or P-selectin expression using flow cytometry. Furthermore, western blot analysis of platelets co-incubated with thrombin and insulin revealed that insulin failed to alter phosphorylation of intracellular platelet activation markers: protein kinase B (PKB), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) (Rauchfuss et al., 2008). It also failed to alter phosphorylation of the intracellular marker of platelet inhibition, VASP. To support this, they showed that insulin did not alter ADP, collagen or thrombin-induced platelet aggregation, nor did it affect calcium mobilisation, confirming that insulin does not have a direct effect on platelet function. Additionally, western blot analysis showed that insulin stimulation causes phosphorylation of the insulin receptor (IR), endothelial nitric oxide synthase (eNOS), and downstream kinases (ERK, p38 MAPK and PKB) in endothelial cells but not platelets (Rauchfuss et al., 2008). The data does not directly prove that IR is not expressed in platelets, but indicates that either there is no functionally active IR expressed in platelets or that the copy number is too low to activate downstream protein kinases. Using flow chamber experiments, Rauchfuss et al. (2008) detected an insulin-dependent increase of endothelial NO production that caused a significant reduction in platelet adhesion, indicating that insulin reduces platelet activation by direct action on endothelial cells rather than platelets.

Haeften et al. (2006) reported that insulin inhibited platelet aggregation in healthy subjects but that this inhibition was reduced in type 2 diabetic patients. Patients with prediabetes or early stages of type 2 diabetes have increased levels of circulating insulin, known as hyperinsulinemia (Kakouros et al., 2011). Presumably, this is the body's compensatory response to insulin resistance. Taking this into consideration, it would be expected that hyperinsulinemia would suppress platelet activity. However, this is not the case in obesity, a common trait in type 2 diabetes. In a study using insulin infused platelets from non-obese healthy participants and obese healthy participants, collagen-stimulated platelet aggregation was significantly reduced in the non-obese group but not the obese group (Westerbacka et al., 2002), indicating that obesity may abolish the inhibitory effect of insulin.

1.9 Dyslipidaemia and platelet dysfunction

Type 2 diabetes is associated with atherogenic dyslipidaemia, and it is characterised by the following lipid abnormalities: elevated low-density lipoprotein-cholesterol (LDL-C), greater number of smaller LDL particles, decreased levels of high-density lipoprotein-cholesterol (HDL-C) and increased triglycerides (Bell et al., 2011).

A recent study consisting of fifty obese type 2 diabetic and fifty obese non-diabetic subjects, showed a significant increase in the levels of serum LDL-C, triglycerides and very low-density lipoprotein-cholesterol (VLDL-C) in the diabetic group (Songa et al., 2015). They also found a significant decrease in serum HDL in the diabetic cohort. There was no significant difference between the levels of total cholesterol. Conversely, the Strong Heart Study identified lower levels of LDL-C in American Indians when compared against non-diabetics from the same population (Howard et al., 2000). Another study examined the lipid profiles of African American and white patients with non-insulin dependent diabetes mellitus (NIDDM) (Cowie et al., 1994). They found that the mean LDL-C was significantly lower in the diabetic African American population when compared to the non-diabetic African American group, but the LDL-C was slightly higher in diabetic subjects in the white population.

For many years, the benefits of intervention on lipoproteins as cardiovascular risk factors in diabetes was uncertain and this was because diabetic people were excluded from trials of lipid-lowering therapies (Viljoen et al., 2017). However, LDL-C is now considered a high-risk factor for cardiovascular disease in diabetic

patients. In the Strong Heart Study, Howard et al. (2000) reported that a 10 mg/dL increase in LDL-C was associated with a 12% increase in cardiovascular risk. In addition to this, a meta-analysis of diabetic patients (n= 18,686) in fourteen randomised trials of statins, showed a 21% reduction in major vascular events per mmol/L reduction in LDL-C (Cholesterol Treatment Collaborators, 2008). However, the same effect was found in non-diabetic people (n=71,370), indicating that LDL-C is a major risk factor for cardiovascular disease but it may not have a direct effect on cardiovascular risk in diabetes per se.

LDL-C is a measure of the total cholesterol content within LDL particles, with regards to both the number of LDL particles and their individual cholesterol content (Wadhera et al., 2016). Circulating LDL particles are able to penetrate the endothelium, become oxidised and promote inflammation (Hadi and Suwaidi, 2007). A persistent elevation of LDL-C is linked to early stage fatty streaks that narrow the lumen and progress to more unstable plaques that can rupture and form a clot. For example, LDL-receptor deficient mice that are unable to clear LDL from the circulation, develop severe atherosclerotic lesions (Véniant et al., 2000).

LDL is a spherical particle which transports esterified cholesterol and triglycerides in a hydrophobic core, surrounded by a monolayer of phospholipids and unesterified cholesterol (Akkerman, 2008). It is enclosed by the apolipoprotein B100 (Apo100) molecule that renders the particle water-soluble. LDL are a heterogeneous collection of particles which vary in size, density and lipid composition (Berneis and Krauss, 2002). It has been shown that increased levels

of small dense, triglyceride-rich, LDL particles have been associated with increased cardiovascular risk (Vergés, 2009). In the Strong Heart Study that found lower levels of LDL-C in the diabetic American Indian population, they also reported significantly elevated small dense LDL (sd-LDL) in the diabetic cohort compared with the non-diabetic subjects (Howard et al., 2000).

Having high levels of sd-LDL increases the risk of cardiovascular disease because this type of lipoprotein cannot be cleared by hepatocytes and remains in the plasma for a longer period. Gaelano et al. (1998) showed evidence that this was because sd-LDL has less affinity for LDL receptors and increased binding to cell surface proteoglycans of the arterial matrix.

Sd-LDL is more susceptible to chemical modification (e.g. glycation or oxidation) and this renders it more atherogenic (Wadhera et al., 2016). Glycation of LDL occurs due to the non-enzymatic reaction of glucose and its metabolites with the free amino acids in lysine-rich LDL (Younis et al., 2008). Higher concentrations of glycated sd-LDL are present in diabetes patients than those without diabetes. Oxidation of LDL occurs by a process involving lipid peroxidation (Parthasarathy et al., 2010). During this process free radicals take electrons from the lipid membranes to form secondary products, resulting in cell damage. The oxidation of LDL is a complex process and many different secondary products can be formed depending on the type of oxidant, the duration of oxidation and the presence or absence of other agents such as redox metals (Parthasarathy et al., 2010). Ox-LDL accumulates in atherosclerotic plaques and there is ~6 fold more ox-LDL in atherosclerotic plaques than in normal intima (Korporaal et al., 2005).

Early studies with animals showed that antioxidants could reduce atherosclerosis. For example, rabbits fed a 1% cholesterol diet with or without the antioxidant butylated hydroxytoluene (BHT) developed typical atherosclerotic lesions in the aorta, though the degree of atherosclerosis was considerably lower in the rabbits fed BHT (Björkhem et al, 1991). However, meta-analysis data for large clinical trials of anti-oxidants such as vitamin E and β-carotene demonstrated no benefit to cardiovascular outcomes (Kris-Etherton et al., 2004). This then prompts the question: is the oxidative modification hypothesis relevant to human disease? Those who have done intensive research into ox-LDL would argue that the optimum antioxidant dosage has simply not been determined, or that the appropriate antioxidant(s) only work in a subset of patients (Steinberg and Witztum, 2010).

The evidence that LDL contributes to atherosclerosis is considerable, and as platelets play a key role in thrombotic complications, it is possible that LDL directly contributes to platelet hyperreactivity in atherothrombosis. Platelets have no nucleus and, thus, are unable to synthesise cholesterol (Colwell et al., 1992). Therefore, the source of this cholesterol must come from either the parent megakaryocyte or the plasma through the interaction with lipoproteins which transport cholesterol. Platelets have an n-LDL receptor, Apolipoprotein E Receptor 2' (ApoER2'), a 130-kDa splice variant of apoER2 (Korporaal et al., 2004). ApoER2' is activated by contact with the apoB100 of LDL, called the B-site. Receptor phosphorylation initiates signalling through p38MAPK and cytosolic phospholipase A_2 leading to formation of TXA_2 (Akkerman, 2008). TXA_2 further activates platelets by stimulating the TXA_2 receptor, leading to $\alpha_{IIb}\beta_3$

activation, and ligand-mediated inside-out signalling by $\alpha_{IIb}\beta_3$ that induces a second wave of activating signals (Akkerman, 2008).

Studies have shown a potential link between the ox-LDL scavenger receptor, CD36, and platelet hyperreactivity. Podrez et al. (2007) used *apoe*$^{-/-}$ / CD36$^{-/-}$ mice to demonstrate that diet-induced hyperlipidaemia promotes platelet hyperreactivity through the uptake of ox-LDL by CD36 on the platelet surface. Magwenzi et al. (2015), however only observed a moderate elevation in platelet reactivity by ox-LDL, but demonstrated that ox-LDL can induce the generation of platelet ROS through CD36 via a NAD phosphate oxidase (NOX) 2 pathway. Furthermore, the NOX2-generated ROS activates platelets indirectly by blocking the ability of cGMP to inhibit platelet function, supporting the notion that platelet hyperreactivity in diabetes is caused by impaired sensitivity to platelet inhibitors.

Nagy et al. (2011) demonstrated that the $P2Y_{12}$ receptor pathway significantly amplified platelet responses of $\alpha_{IIb}\beta_3$ in LDLR$^{-/-}$ mice with elevated cholesterol levels. Korporaal et al. (2005) investigated the association of both native-LDL (n-LDL) and ox-LDL with CD36 in platelets, and its effect on $\alpha_{IIb}\beta_3$ activation. They reported that n-LDL binds CD36 for <5 mins and ox-LDL is continually associated with CD36. Interestingly, they reported an inhibition in $\alpha_{IIb}\beta_3$ activation when LDL was oxidised >30%. CD36 is known to associate with $\alpha_{IIb}\beta_3$ on resting platelets (Dorahy et al., 1996) and co-localise with $\alpha_{IIb}\beta_3$ and fibrinogen on activated platelets (Asch et al., 1985). Korporaal et al. (2005) determined that binding of ox-LDL to CD36 blocks binding of CD36 to $\alpha_{IIb}\beta_3$, potentially interfering with fibrinogen-bound $\alpha_{IIb}\beta_3$activation. It is unclear how CD36 is facilitating $\alpha_{IIb}\beta_3$

activation, however, Kuijpers et al. (2014) reported an anchoring role for CD36 and its ligand, thrombospondin-1 (a protein released from α-granules), contributing to the stability of a thrombus.

Hyperlipidaemia often accompanies hyperglycaemia in patients with type 2 diabetes. To investigate if hyperglycaemia and/or hyperlipidaemia are responsible for the prothrombotic state in type 2 diabetes, Zhu et al. (2012) used a type 2 diabetic mouse model (*DBD*) fed a high fat/high fructose diet to induce both hyperlipidaemia and hyperglycaemia, and a control group fed a western diet to induce hyperlipidaemia but not hyperglycaemia (*WD*). Interestingly, they found that the time to carotid thrombosis was considerably shorter in the *DBD* mice than the *WD* mice, suggesting that hyperglycaemia is responsible for the prothrombotic state in the *DBD* model, possibly due to lipoprotein modification. They also found that a CD36 deletion rescued the carotid thrombi phenotype, supporting other studies that demonstrate the involvement of CD36 in thrombus formation.

Altered lipid profiles in type 2 diabetic patients has also been demonstrated to impair bioavailability of endothelial-derived platelet inhibitors. Studies have shown that endothelial NO and PGI_2 bioavailability is affected by ox-LDL which accumulates in the arterial wall. An early study showed that increased levels of ox-LDL interferes with the cAMP and cGMP pathways in isolated arteries (Galle et al., 1992). Furthermore, Wang et al. (2011) showed that ox-LDL upregulates arginase I which reduces l-arginine availability for eNOS, and this, in turn, compromises NO synthesis since arginase and NO share the substrate l-arginine.

These findings would suggest that the accumulation of modified lipoproteins in the endothelium causes impaired bioavailability of NO and PGI_2, which ultimately leads to increased platelet activation.

Other literature has shown that cholesterol accumulation disturbs the composition of membranes of hematopoietic cells, particularly in lipid rafts, and this enhances receptor signalling (Zhu et al., 2010; Yvan-Charvet et al, 2010). This may be important in regards to altered platelet signalling in diabetic patients and efficacy of antiplatelet medication. For example, $P2Y_{12}$ has been shown to be located in lipid rafts (Quinton et al., 2005) and increased expression of $P2Y_{12}$ has been reported in diabetic patients (Hu et al., 2017). Thus, plasma membrane cholesterol accumulation in platelets could potentially alter the membrane structure and affect signalling via surface receptors, such as $P2Y_{12}$. Furthermore, this may contribute to antiplatelet resistance in type 2 diabetes.

Although considerable data has concentrated on LDL-C as a potential atherogenic marker, Chan et al. (2014) investigated the relationship between both LDL-C and HDL-C with platelet reactivity in hyperlipidaemic patients. They identified a significant increase in P-selectin expression and $\alpha_{IIb}\beta_3$ activation in patients with high LDL-C and low HDL-C, but no difference in patients with high LDL-C and normal HDL-C. This suggests that the cardio-protective properties of HDL-C have an effect on platelet reactivity.

Triglycerides are formed from a single molecule of glycerol combined with three fatty acids (Viljoen et al., 2017). The two main sources of triglycerides are

exogenous (from dietary fats) carried in chylomicrons produced by the gut and endogenous sources carried by VLDL produced by the liver. A meta-analysis of 262,525 participants found a strong association between serum triglyceride levels and cardiovascular risk in Western populations (Sarwar et al., 2007).

Measurement of platelet aggregation with blood from hypertriglyceridemia patients cannot be implemented due to the turbid plasma samples, however, whole blood flow cytometry can be performed (De Man et al., 2000). Although no literature is available to show the relationship between triglycerides and platelet reactivity in diabetic patients, a small study (n=16) reported no association between high triglycerides and both P-selectin expression and $\alpha_{IIb}\beta_3$ activation in hypertriglyceridemia patients (De Man et al., 2000).

In conclusion, elevated LDL may contribute to platelet hyperreactivity in cardiovascular disease. However, thus far, no research has been obtained to link platelet activation with LDL metabolism in the type 2 diabetic population.

1.10 Obesity, Inflammation and Platelet dysfunction

According to the World Health Organisation (2017) obesity is defined as BMI $\geq$ 30 kg/m^2 and, at present, the prevalence of obesity is in the range of 15% to 35% in the adult populations of Europe, North America and many Arabic countries. Patients become insulin resistant almost always because of obesity and physical inactivity (Nussey and Whitehead, 2001). A recent analysis of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort revealed that a weight gain of 1 BMI unit between the age of 24 to 40 years increased the relative risk of type 2 diabetes by 25%, and, surprisingly, they were more at risk than those between the age of 40 and 55 years (Schienkiewitz et al., 2006). This indicates that weight gain at early adulthood is related to a higher risk and earlier onset of type 2 diabetes, than is weight gain between 40 and 55 years of age.

A small number of studies have investigated the relationship between obesity and platelet reactivity in type 2 diabetic patients. Akinsegun et al. (2014) identified a positive relationship between BMI and platelet count in type 2 diabetic patients, but no significant association between BMI and MPV. Schneider et al. (2009b) reported a correlation with BMI and ADP-induced P-selectin expression in type 2 diabetic patients with CAD, but no correlation with $\alpha_{IIb}\beta_3$ activation.

As previously mentioned, platelet activation is counteracted by the inhibitors NO and PGI_2, which act by increasing intracellular levels of cGMP and cAMP. Early studies reported that insulin had an anti-aggregatory effect on platelets by increasing the levels of cGMP and cAMP (Trovati et al., 1997). Interestingly the

same team identified that the anti-aggregating effect of insulin, mediated by NO-induced increase of cGMP, was lost in obese subjects and obese subjects with type 2 diabetes but not lean type 2 diabetic (Anfossi et al., 1998).

Obesity is commonly associated with low-level inflammation and insulin resistance. In 1993, Hotamisligil and co-workers identified the link between obesity and inflammation when it was shown that the inflammatory cytokine, tumor necrosis factor (TNF)-α, was overexpressed in the adipose tissue of a rodent model for obesity. Diabetes often accompanies obesity, and Hotamisligil et al (1993) also found that obese mice lacking functional TNF-α had a significant increase in uptake of glucose by insulin. Once this discovery was made, it became clear that many inflammatory mediators respond in a similar manner to TNF-α in animal models of obesity (Wellen and Hotamisligil, 2005).

A mechanism that may contribute to chronic inflammation in diabetes is oxidative stress (Wellen and Hotamisligil, 2005). Due to the increased delivery of glucose to the adipose tissue, endothelial cells in the fat pad may take up increasing amounts of glucose under hyperglycaemic conditions. These endothelial cells produce excess ROS in their mitochondria, which activates metabolic events in diabetes such as increased AGEs, PKC and NF$\kappa\beta$ activation (De Bandiera et al., 2013). The binding of AGEs to endothelial cells may activate NF$\kappa\beta$ to induce expression of the leukocyte adhesion molecules vCAM-1, ICAM-1 and E-selectin (Basta e al., 2002). Furthermore, the gene regions NF$\kappa\beta$ and IL-6 are located at the RAGE promoter region, indicating that the expression of the RAGE receptor

influences inflammatory responses (De Bandiera et al., 2013, Nonaka et al., 2018).

The subsequent activation and recruitment of inflammatory cells, including monocytes and macrophages, results in enhanced cytokine synthesis and release (Lumeng et al., 2007). These cytokines, including TNF-α, interleukin (IL)-β1 and IL-6; can downregulate cascades involved in insulin signalling in adipocytes, liver and muscle cells, enhancing insulin resistance and impaired glucose homeostasis (Badawi et al., 2010). Increased levels of these pro-inflammatory cytokines lead to hepatic production and secretion of acute-phase proteins such as C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1) (Badawi et al., 2010). These proteins appear in the early stages of type 2 diabetes, and their circulating concentrations increase as the disease progresses. For example, concentrations of IL-6, PAI-1, CRP, and fibrinogen were significantly higher in healthy subjects who became diabetic later in life (after 4–10 years) compared to those who did not develop the disease (Pradhan et al., 2001). Moreover, levels of pro-inflammatory cytokines (e.g. IL-6, IL-12, TNF-α), endothelial dysfunction markers (vascular cell adhesion molecule-1 (vCAM-1), intercellular adhesion molecule-1 (ICAM-1), and nitric oxide), and dyslipidaemia were highest in type 2 diabetic patients with cardiovascular complications (Mishra et al., 2011). Conversely, Al-Shukaili et al. (2013) reported decreased levels of the proinflammatory markers IL-6, IL-β1 and TNF-α in fifty-seven type 2 diabetic patients compared to thirty controls, with no difference in the levels of lymphocytes. Although there is conflicting data with regards to

cytokine levels in type 2 diabetic patients, there does appear to be an alteration in the function of the immune system in these patients.

Diabetic patients exhibit increased markers of both platelet activation and inflammation (Lim et al., 2004). Furthermore, studies have demonstrated that the serum level of chemokines can predict the development of atherosclerosis (Ardigo et al., 2007) and also predict acute cardiovascular events (Kraaijeveld et al., 2007). Accumulating research has identified platelets as key players in (thrombo-) inflammatory processes. In the mid 1990's it was shown that resting and activated platelets could adhere to the vessel wall without vessel injury (Frenette et al., 1995). Furthermore, they showed that activated platelets preferentially bound leukocytes and this was dependent on the expression of endothelial P-selectin. In apoE deficient mice, it was demonstrated that platelets could bind to the endothelium before leukocyte invasion and before the development of atherosclerotic lesions (Massberg et al., 2002). Moreover, antibody blockade of GP1bα prohibited leukocyte accumulation and decreased atherosclerotic lesion formation.

Using intravital microscopy in mice treated with TNF-α, neutrophils were visualised migrating to the walls of inflamed blood vessels (Sreeramkumar et al. 2014). This was shown to be mediated by the platelet P-selectin receptor and PSGL-1 on neutrophils. Blocking this interaction reduced the risk of stroke in a mouse model for acute lung injury, highlighting the antithrombotic potential of PSGL-1.

As platelets move through the vasculature of inflamed tissue, they are exposed to a number of different inflammatory mediators such as lipid mediators, cytokines and chemokines released by activated leukocytes, endothelial cells and perivascular cells (Stokes and Granger, 2012). These mediators attach to platelet receptors to elicit dense and/or a-granule secretion and activation of platelet adhesion molecules. Soluble mediators that can activate platelets are lipid mediators (e.g. platelet activating factor (PAF)), cytokines (e.g. interferon-γ (INF-γ), interleukin-2 (IL-2) and chemokines (e.g. CXCL12, CCL22) (Gleissner et al., 2008).

In addition to this, platelets can themselves initiate inflammatory responses through their receptors (Vieira-de-Abreu et al., 2012). For example, thrombin stimulates the activation of the mammalian target of rapamycin (mTOR) in platelets (Moore at al., 2014). The mTOR cascade mediates major immune responses, such as the activation and proliferation of T-cells (Thomson et al., 2009), suggesting that thrombin may have unrecognised functions in immunity (Vieira-de-Abreu et al., 2012). The antagonist of the ADP receptor, clopidogrel, was reported to inhibit platelet-leukocyte adhesion (Evangelista et al., 2005), indicating that the $P2Y_{12}$ pathway may be involved in platelet-dependent upregulation of inflammatory functions. Lastly, it has been shown that T-cell proliferation is reduced in mice with a deficient TXA_2 receptor (Thomas et al., 2003).

Several hormones and inflammatory mediators influence the release of platelets from megakaryocytes in the bone marrow including thrombopoietin (Sato et al.,

1998) IL-1β and IL-6 (Jiang et al., 1994). Studies have shown that diabetic patients with nephropathy have increased platelet count (Sterner et al., 1998) and diabetic patients also have increased levels of immature platelets (Mijovic et al., 2015). Platelet production is regulated to meet the demands for activated platelets in certain physiological conditions. In inflammatory disorders, the larger, more reactive platelets migrate to inflammatory sites where they are consumed, and this enhances thrombopoiesis to increase the quantity of circulating platelets. This results in a shift in platelet indices such as MPV and platelet count (Gasparyan et al., 2011).

Evidence has accumulated suggesting an important role of MPV as a marker of inflammation because the size of circulating platelets is dependent on the intensity of the systemic inflammation. For example, it was reported that patients with systemic lupus erythematosus and rheumatoid arthritis patients have low MPV at the active stage of the disease (Turner-Stokes et al.,1991; Gasparyan et al., 2010) and it is thought that this is due to the increased consumption of platelets at the sites of inflammation (Gasparyan et a., 2011).

Anti-platelet therapy has been important in preventing cardiovascular events in high-risk patients, including those with diabetes. Potentially combining anti-platelet therapy with anti-inflammatory medication can further improve cardiovascular therapies in diabetic patients.

1.11 Research Aims and Objectives

Previous research demonstrates that type 2 diabetic patients have hyperreactive platelets (Kakouros et al., 2011). However, it is not clear which of the biochemical factors related to the disease are responsible, such as hyperglycaemia, insulin resistance, chronic inflammation or elevated cholesterol levels. It is also unclear whether platelet hyperreactivity in diabetes is intrinsic to the platelet or a consequence of endothelial dysfunction or reduced responsiveness to platelet inhibitors.

The primary aim of this study was to systematically investigate both the direct and indirect mechanisms that contribute to platelet dysfunction in type 2 diabetes and identify the key risk factor associated with enhanced platelet reactivity.

The first objective was to examine the acute effects of hyperglycaemia on platelet function using *in vitro* methods. In addition, the indirect effects of hyperglycaemia were investigated by looking at the inhibitory effect of NO and PGI_2 on platelet function in the presence of high glucose.

The second objective was to investigate the relationship between platelet reactivity and clinical parameters (e.g. hyperglycaemia, cholesterol levels and BMI) in type 2 diabetic patients using *ex vivo* methods.

The third objective was to investigate the relationship between inflammatory markers and platelet reactivity in type 2 diabetic patients, to determine whether there is an association between chronic inflammation and platelet hyperreactivity.

The final objective was focused on the comparison between different biochemical parameters and platelet reactivity in three separate cohorts: control, hyperlipidaemic and type 2 diabetic. The control cohort were not taking medication related to diabetes and dyslipidaemia. The hyperlipidaemic cohort were statin-treated for elevated lipid levels but not diagnosed as diabetic. The third cohort were type 2 diabetic patients taking statin medication for dyslipidaemia. The purpose of this objective was to determine if the platelet hyperreactivity is associated with the diabetic condition per se, or whether platelet hyperreactivity is caused by other mechanisms not associated with hyperglycaemia.

2 Methods

2.1 Patient Recruitment

2.1.1 Study population

Healthy participants were used for all *in vitro* experiments in chapter 3 to evaluate the effect of platelet function in high glucose conditions, without the influence of a pre-existing medical condition or medication which could trigger or cause platelet dysfunction. These include antiplatelet drugs (e.g. ADP antagonists and $\alpha_{IIb}\beta_3$ receptor blockers), cardiovascular and lipid-lowering drugs, and nonsteroidal anti-inflammatory medication (Scharf, 2012). Medical conditions that affect platelet function include inherited platelet disorders (e.g. Von Willebrand disease and Glanzmann's thrombasthenia) (Handin, 2005) and acquired platelet disorders caused by disease (e.g. cirrhosis, lupus, renal disease, diabetes and coronary heart disease) (Casari and Bergmeier, 2016). Other exclusion criteria included transmittable bloodborne diseases e.g. HIV/AIDS and hepatitis. A medical questionnaire was given to participants before recruitment to ensure no pre-existing medical condition or medication (see appendix 9.3.9 for the questionnaire). Healthy participants were mainly recruited from Manchester Metropolitan University. Ethical approval for this study was given by the Ethics Committee at MMU. Inclusion criteria included participants between 18-80 years of age. FBG was measured to ensure that they were within the healthy range 3.5-5.5 mmol/L (Tuch et al., 2000).

For chapters 4 and 5, type 2 diabetic patients were recruited from the Diabetes Centre at the Manchester Royal Infirmary (MRI). Platelets from the type 2 diabetic cohort were investigated for correlations against risk factors associated with the

disease. Patient screening was carried out using DIAMOND, a diabetes monitoring database. Inclusion criteria comprised type 2 diabetes, between 18-80 years old. All patients recruited for the diabetic arm of the study were diagnosed and being treated for type 2 diabetes. Exclusion criteria included disorders/medication that could affect platelet function: type 1 diabetes; coronary heart disease (such as angina and a previous myocardial infarction); those receiving anticoagulant therapy; use of nonsteroidal inflammatory drugs (NSAIDs); those taking aspirin or other antiplatelet medication; bleeding disorders such as Von Willebrand's disease. Other exclusion criteria included the transmittable bloodborne diseases (HIV/AIDS and hepatitis). Also excluded were those with severe hypertriglyceridemia (triglycerides >4.5 mmol/L) because the high lipid content in plasma can affect platelet aggregometry tests. Kidney disease is common in diabetes and it can affect platelet function (Boccardo et al., 2004), so creatinine levels were measured to ensure that kidneys were functioning properly (normal range 45-90 μmol/L). Medical history and current medications were identified using DIAMOND.

For chapter 6, a control group and a hyperlipidaemic group were used to compare platelet reactivity with a diabetic group taking lipid-lowering medication. All diabetic participants had the same exclusion/inclusion criteria as above, except that lipid lowering medication was included in the inclusion criteria. The control group were used as a comparison group that was not diabetic and not on lipid-lowering medication. The hyperlipidaemic group were used as a comparison group that was not diabetic and was taking lipid-lowering medication. This was to identify whether the lipid lowering medication influenced platelet reactivity,

independent of diabetes. Both groups were age-matched to the diabetic group as closely as possible as age can affect platelet function (Jones, 2016). HbA1c and FBG was measured in both cohorts to ensure that they were not diabetic (HbA1c >47.0 mmol/mol or fasting blood glucose >7.0 mmol/L) or prediabetic (HbA1c >42.0 mmol/mol or fasting blood glucose >5.6 mmol/L). Creatinine levels were also measured to ensure that the kidneys were functioning properly (normal range 45-90 μmol/L) as renal disease can affect platelet function (Boccardo et al., 2004). Additionally, chronic renal failure is associated with shortened red blood cell survival and this could lower HbA1c results (Peacock et al., 2008).

Hyperlipidaemic patients were recruited from the Lipid Clinic held at the Outpatients Department in the MRI. Patient screening was carried out using Medisec and Chameleon databases. Inclusion criteria comprised patients with a diagnosis of hyperlipidaemia, between 18-80 years old, and those taking lipid-lowering medication.

For the control and hyperlipidaemic participants, exclusion criteria included: diabetes; coronary heart disease (such as angina and a previous myocardial infarction); those receiving anticoagulant therapy; use of nonsteroidal inflammatory drugs (NSAIDs); those taking aspirin or other antiplatelet medication; bleeding disorders such as haemophilia and Von Willebrand's disease; HIV/AIDS; severe hypertriglyceridemia (triglycerides >4.5 mmol/L). This was identified using the hospital clinic notes for hyperlipidaemic patients, and was identified using a questionnaire (see appendix 9.3.9) for the control participants.

2.1.2 Ethics

For healthy participants recruited from MMU for the *in vitro* studies (chapter 3), ethical approval was given by the Ethics Committee at MMU.

For type 2 diabetic participants, hyperlipidaemic and healthy controls used for chapter 4, 5 and 6, ethical approval for this study was given by the NHS Ethics Research Service (REC Reference: 11/NW/0731). Patient Information sheets were posted to patients and healthy controls at least 24 hours prior to consent (see appendix, section 9.3, for patient information sheets, cover letters and consent forms). Informed consent was obtained from all participating subjects.

2.2 Sample Preparation

2.2.1 Venepuncture

Blood was collected from healthy participants at Manchester Metropolitan University (MMU) according to a protocol approved by the local ethical committee of MMU. Healthy subjects were instructed to fast overnight and blood was drawn between 8.00am and 10.00am. Blood was obtained from diabetic and hyperlipidaemic patients on the day of their appointment at the Diabetes Centre or Lipid Clinic. Patients were instructed to fast for 12 hours before the hospital appointment.

Blood was drawn from the median cubital vein of rested subjects using a 21-gauge needle (BD Vacutainer). Blood was collected into BD Vacutainer tubes (BD, Manchester, UK) containing citrate (3.2%), dipotassium ethylenediaminetetraacetic acid (K2 EDTA) and a serum tube with gel separator. Platelets are easily activated, so blood was drawn with minimum trauma. The tubes were gently inverted 3-4 times to mix the blood with the anticoagulant. Blood specimens were maintained at 20°C. Testing was performed up to 3 hours after venepuncture.

2.2.2 Preparation of Serum and Plasma for Immunoassays

Blood collected from healthy and patients (as described in section 2.3) was centrifuged at 1500 x g for 10 minutes at 4ºC. Plasma (from citrate and EDTA tubes) and serum were stored as 0.5 ml aliquots at -80ºC.

2.2.3 Preparation of PRP

PRP was obtained by centrifugation of the blood (from sodium citrate tubes) at 180 *g* for 10 min at 20^{o}C. PRP was diluted (1:1) in modified tyrodes-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12mM $NaHCO_3$, 1mM $MgCl_2$, 20mM HEPES, 5mM glucose). Platelet count and MPV was measured for the diluted PRP, as outlined in section 2.3. Platelet poor plasma (PPP) was obtained by centrifugation of PRP at 1800 *g* for 5 minutes at 20^{o}C.

2.3 Biochemical Analysis

2.3.1 Full Blood Count

Blood was collected into K2 EDTA tubes and platelet parameters measured within 1 hour after venepuncture to avoid bias due to excessive platelet swelling. Blood specimens were gently inverted 5 times. A full blood count was measured using an automated blood analyser (Sysmex XS 1000*i*^{TM}, UK). The volume and structures of platelets in the blood are heterogenous, so the following haematological parameters were studied in all blood samples: platelet count, mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR). For MPV, the blood analyser measures the platelet volume directly by calculating the log transformation of the platelet volume distribution curve to yield a geometric mean (Budak et al., 2016). PDW is an indicator of volume variability in platelet size. It calculated using a distribution curve of platelets measured at the level of 20% relative height in a platelet-size distribution curve, with a total curve height of 100% (Budak et al., 2016). P-LCR is an indicator of circulating larger platelets (>12 fL), which is presented as percentage (Budak et al., 2016).

2.3.2 Glucose Measurements

For blood glucose readings, 10 µl of K2 EDTA blood was analysed according to the manufacturer's instructions (Accu-Chek Aviva, Roche Diagnostics Ltd, East KJKJH, UK). HbA1c was measured in EDTA blood using a haemoglobin testing system (BIO-RAD Variant II).

2.3.3 Lipid Profile

Lipids and creatinine were measured in serum using an autoanalyser (Roche COBAS 701).

2.4 Aggregometry

2.4.1 Light transmission aggregometry

Light transmission aggregometry (LTA), designed by Born in the 1960s (Born, 1962) is deemed the gold standard in platelet function testing. This test assesses the platelet-to-platelet clump formation in a $\alpha_{IIb}\beta_3$ manner, known as aggregation (Paniccia et al., 2015). The assay is based on the measurement of light transmission through the optically dense PRP after the addition of an agonist. As the platelets are activated and aggregate, the plasma becomes clearer and the light transmission increases. The device records the rate and maximal percentage of this increase from 0% (maximal optical density of PRP) to 100% (no optical density of autologous platelet-poor plasma) by a photometer. This signal is converted automatically in a graphic curve (Paniccia et al., 2015).

LTA was performed for the *in vitro* experiments in chapter 3 and the *ex vivo* correlation data in chapters 4,5, and 6. For the *in vitro* experiments, PRP was incubated with 0, 5, 15 and 30 mmol/L D-glucose (Sigma, Poole, UK) at room temperature and 37^{o}C for one hour prior to platelet aggregation testing.

For dose response curves, PRP was stimulated with 1-10 µmol/L ADP (Sigma, Poole, UK), and 1-4 µg/ml collagen (Labmedics, Manchester, UK) at 37^{o}C under constant stirring conditions. EC_{50} values (the concentration necessary to induce half-maximal aggregation) were obtained from non-linear regression curves (Appendix 9.1).

For the in vitro experiments (chapter 3), platelet aggregation was measured in response to submaximal concentrations of ADP (2.5 µmol/L) and collagen (1.0 µg/ml), elucidated from a dose response (appendix 9.1). For the *ex vivo* experiments (chapters 4, 5 and 6), platelet aggregation was measured in response to submaximal concentrations of ADP (5.0 µmol/L) and collagen (1.0 µg/ml), elucidated from a dose response (appendix 9.1).

To measure the maximum aggregation in the presence of platelet inhibitors, a dose response was performed in PRP using Sodium Nitroprusside (SNP) (Sigma, Poole, UK) and Prostaglandin E_1 (PGE_1) (Sigma, Poole, UK) to obtain a concentration that induces almost complete inhibition (appendix 9.1). For the experiment SNP (100 µM) PGE_1 (0.25 µM) inhibitors were incubated in PRP for 2.5 minutes at 37ºC before the agonist was added. PRP was stimulated with ADP (2.5 µmol/L) and collagen (1 µg/ml).

The aggregation tracing was observed for 5 min using a Chronolog Model 700 Aggregometer (Haverton, PA, USA). The maximum aggregation (%) was displayed using an AGGRO/LINK8 program (Chronolog).

2.4.2 Impedance aggregometry

Impedance whole blood aggregometry allows one to assess platelet function by using the anticoagulated whole blood as milieu without any sample processing (Paniccia et al., 2015). It is based on the principle that activated platelets stick via their surface receptors to artificial surfaces of two electrodes within the whole

blood sample positioned at a determined distance between them. Platelet aggregation is assessed by detecting the increase in electrical impedance generated by the aggregation of other platelets upon those fixed to the electrodes. The degree of the increase in impedance is recorded in ohms as a graphic curve (Paniccia et al., 2015).

For *in vitro* experiments (chapter 3), whole blood was incubated with D-glucose (0, 5, 15 and 30 mmol/L) at room temperature and 37ºC for one hour prior to platelet aggregation testing.

Whole blood was diluted (1:1) with pre-warmed tyrodes-HEPES buffer and incubated at 37ºC for 5 min. For dose response curves, blood was stimulated with ADP (1-20 µmol/L) and collagen (0.5-10 µg/ml) at 37ºC under stirring conditions. EC_{50} values (the concentration necessary to induce half maximal aggregation) were obtained from concentration-effect curves (appendix 9.1). For glucose experiments, blood was stimulated with ADP (2.5 µmol/l) and collagen (1 µg/ml). The aggregation tracing was observed for 5 min using a Chronolog Model 700 Aggregometer (Haverton, PA, USA). The maximum aggregation (ohms) was displayed using an AGGRO/LINK8 program (Chronolog, USA).

2.4.3 Lumi-aggregometry

Lumi-aggregometry allows simultaneous measurement of the release of adenine nucleotides from platelet granules and platelet aggregation (Paniccia et al., 2015). The method is based on the measurement of ATP released from activated

platelets by different agonists by using a luminescence technique in plasma or whole blood. The assay is based on the conversion of ADP, released from the platelet dense granules, to ATP that reacts with the luciferin–luciferase reagent. The light emitted, proportional to the ATP concentration, is quantified by the lumi-aggregometer (Chronolog Model 700 Aggregometer) (Paniccia et al., 2015).

Prior to testing, ATP release was calibrated using an ATP Standard (Labmedics, Manchester, UK), according to manufacturer's instructions (Chronolog, USA). 25 µl CHRONO-LUME™ (Labmedics, Manchester, UK) was added to 475 µl of pre-warmed PRP. The CHRONO-LUME/PRP was incubated at 37^{o}C for 2 min, before the addition of ADP (2.5 µmol/l) and collagen (1 µg/ml). ATP release (nmoles) was displayed against platelet aggregation (%) using an AGGRO/LINK8 program (Chronolog, USA).

2.5 Flow Cytometry

2.5.1 Measurement of Platelet Adhesion Molecules

Flow-cytometric measurement of platelet surface glycoproteins in fixed whole blood is a sensitive and quantitative analysis of platelet activation (Goodhall and Appleby, 2004). It involves the rapid analysis of thousands of individual cells per second as they pass through a laser light source. Reflected light is translated into an electronic signal, which represents a measure of the cells' size and granularity based on forward scatter and side scatter, respectively. This can identify the different cell types using their distinct morphological characteristics within whole blood. Thus, platelets can be identified without the need for separation from the other blood cells (Goodhall and Appleby, 2004). Additionally, whole blood can be preincubated with fluorescently-labelled antibodies that recognise antigens on the platelet surface. The flow cytometer can measure the number of fluorescent antibodies on each cell, which is directly proportional to the number of antigens on the platelet surface. Fluorescence measurement can be expressed as either the % of positive cells above a threshold set with an appropriate negative control, or as the mean or median fluorescent intensity (MFI) of a population of cells (Goodhall and Appleby, 2004).

In this study, a dual-fluorescent assay was used. In which, platelets were co-labelled with an antibody that recognises the fibrinogen-binding site on the activated $\alpha_{IIb}\beta_3$ receptor (PAC-1 antibody) and an antibody for α-granule secretion (CD62P antibody).

Aliquots (5µl) of whole blood were added to 45 µl of tyrodes buffer containing 5µl fluorescent monoclonal antibodies, PE Mouse Anti-Human CD62P (BD Biosciences) and PAC-1 FITC (BD Biosciences). An aliquot (5µl) of APC Mouse Anti-Human CD42b (BD Biosciences) was added to all samples for platelet identification in the whole blood. The CD42b antibody recognises the GPIbα in platelets. The samples were incubated in the dark at room temperature for 20 minutes with vehicle or agonist (5.0 µmol/L ADP). For the *in vitro* experiments (chapter 3) samples were also incubated with 0, 5, 15 or 30 mmol/L glucose. To measure the quantity of platelet adhesion molecules in the presence of platelet inhibitors, SNP (100 µM) and PGE1 (0.25 µM) was added to the samples. After incubation, the samples were diluted and mildly fixed with 0.4% (v/v) paraformaldehyde and phosphate buffer solution (PBS) before analysis using a BD FacsVerse™ flow cytometer (BD Biosciences). Platelets were identified on their logarithmic side scatter and CD42b positivity expression. Analysis was performed with computer software (BD FACSuite™ Software).

The PE (CD62P antibody) and FITC (PAC-1 antibody) fluorochromes are excited at neighbouring channels which can lead to 'bleed-through'. This occurs when the signals of the two fluorescence antibodies are detected in the same channel. To avoid this from occurring, the flow cytometer was set up so that the PE and FITC signals were not overlapping. Quadrant gates were set up to divide the populations into $CD62P^-/PAC\text{-}1^-$, $CD62P^+/PAC\text{-}1^-$, $CD62P^-/PAC\text{-}1^+$ and $CD62P^+/PAC\text{-}1^+$ (see figure 9.10 in appendix). Any bleed-through was removed by adjusting the photomultiplier tube voltage.

For the SNP and PGE_1 *in vitro* flow cytometry experiments (chapter 3) the % inhibition (of basal) was determined as follows:

Four samples were set up for each experiment: non-stimulated whole blood, non-stimulated whole blood + Inhibitor, ADP-stimulated whole blood, ADP-stimulated whole blood + Inhibitor. The % inhibition was calculated as follows:

= Stimulated (MFI) – Non-stimulated (MFI) = *x*

= Stimulated with inhibitor (MFI) – Non-stimulated with inhibitor (MFI) = *y*

= (*y*/*x*) *100

= % Inhibition (of basal conditions)

2.5.2 Immature Platelet Fraction

Newly formed platelets, although anucleated, contain mRNA and are able to synthesize small amounts of protein (Robinson et al., 1998). They are called reticulated platelets due to the staining pattern of the cytoplasmic mRNA distribution (Mijovic et al., 2015). The measurement of reticulated platelets has considerable clinical utility for monitoring thrombopoiesis and platelet turnover (Robinson et al., 1998). Thiazole orange (TO) is a fluorescent nucleic acid dye used for flow cytometric detection of reticulated platelets (Robinson et al., 1998).

Venous blood samples anticoagulated with K2 EDTA, were kept at room temperature until analysis was performed, less than 4 hours after blood collection. 5 μl of whole blood was added to 1 ml of TO (BD Retic-Count™, BD Biosciences, US). A negative control was used for each sample, and prepared using 5 μl whole blood and 1 ml of PBS with 0.1% sodium azide. All samples were labelled with 5

μl APC Mouse Anti-Human CD42b (BD Biosciences, US) for the identification of platelets among the other blood cells. Samples were incubated for 30 min at room temperature in the dark. They were read in the cytometer (BD FACSVerse™, BD Biosciences, US). Platelets were identified on their logarithmic side scatter and CD42b positivity expression. Analysis was performed with computer software (BD FACSuite™ Software). A dot plot (CD42b-APC vs. TO fluorescence) was generated, and the reticulated platelet rate was expressed as a percentage of both TO and CD42b-APC positive population among 10,000 identified platelets. The threshold of TO fluorescence was chosen so that >99% of the CD42b-APC positive population was negative for TO. The number of positive events in the negative control was subtracted from the number of positive events in the sample. Reticulated platelets were expressed as mean % of TO-positive platelets.

2.6 Immunoassays

2.6.1 ELISA

A commercially available sandwich ox-LDL enzyme-linked immunosorbent assay (ELISA) kit (LifeSpan BioSciences, Inc., Seattle, USA) was used for the quantitative detection of human ox-LDL in EDTA plasma samples from type 2 diabetic patients, previously stored at -80ºC and thawed on ice. The protocol was carried out according to the manufacturer's instructions (LifeSpan BioSciences, Human ox-LDL / Oxidised LDL ELISA kit, catalogue no. LS-F23411). An initial experiment was undertaken to determine the optimum sample dilution factor. This was to ensure that the protein concentration for each sample fell within the range of Optical Density (OD) values distinguished by a standard curve (detection range for the ELISA kit was 31.25-2000 pg/ml). The optimisation experiment determined that the plasma samples should be undiluted for the ELISA assay. Absorbance was measured at 450nm using a microplate reader. To quantify the protein concentration, a standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and a line of best fit curve was drawn (see appendix 9.2 for the standard curve). To measure the extent of oxidation, the ox-LDL and LDL-C values were both converted to mg/dL, and the ox-LDL was divided by the LDL-C to get the ox-LDL/LDL-C ratio.

2.6.2 Luminex Assay

Multiplex Bead Array Assays technology, enable simultaneous detection and quantitation of multiple secreted proteins (cytokines, chemokines, growth factors, etc.) (Elshal and McCoy, 2006). This high-throughput technology produces results comparable to ELISA but with potential cost and time-saving. Thus, a multiplex assay, using Luminex xMAP (multi-analyte profiling) technology, was used for the analysis of inflammatory markers and platelet reactivity due to the high number of cytokines/chemokines involved in inflammation. The Luminex xMAP technology uses digital signal processing capable of identifying polystyrene beads (microspheres) dyed with designated proportions of red and near-infrared fluorophores (Elshal and McCoy, 2006). These microspheres are coated with specific antibodies, which can be read by flow cytometry because the microspheres have distinguishable fluorescent signatures. Up to one hundred different detection reactions can be carried out simultaneously on the various bead populations in very small sample volumes.

Plasma (3.2% citrate) was thawed on ice and centrifuged at 10,000 x g for 10 minutes to remove particulates. The multiplex assay used for this study was the Inflammation 20-Plex Human ProcartaPlex™ Panel (ThermoFisher Scientific, US). The manufacturer recommended that plasma samples not be diluted prior to assay performance due to the low-level cytokine concentrations in human plasma. Protocol was performed as described according to the ProcartaPlex™ Multiplex Immunoassay (ThermoFisher Scientific, US) user guide. The magnetic bead panel was able to simultaneously quantify E-selectin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon(IFN)-α, IFN-γ,

interleukin (IL)-1α, IL-1β, IL-10, IL12p70, IL-13, IL-17A, IL-4, IL-6, IL-8, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, P-selectin, soluble intercellular adhesion molecule (sICAM)-1, tumour necrosis factor (TNF)-α in a 96-well plate. Standards were prepared as outlined in the ProcartaPlex™ Multiplex Immunoassay (ThermoFisher Scientific, US) user guide. The standards contained a mix of multiple proteins, detailed in the Certificate of Analysis document that came with the kit. The protocol included an overnight incubation at 4ºC to improve assay sensitivity.

The plate was analysed on the Luminex 200™ Multiplex instrument. Prior to running the assay, calibration of the Luminex 200™ instrument was undertaken using a Luminex 200™ Calibration kit (ThermoFisher, US) in full accordance with the manufacturer's instructions. Additionally, the probe height was calibrated with a 96-well flat bottom plate supplied with the kit. Information provided in the Certification of Analysis was used set up the Luminex Acquisition Software™ prior to running the assay. These included details of bead region and standard concentrations (pg/ml) for each analyte. Protocol was performed as described according to the Luminex® 200™ System User Manual. The concentration of the analytes was calculated from the standard curve using the Luminex™ xPONENT® software (Luminex™ version 3.1.871).

2.7 Statistical Analysis

Data is presented as mean ± standard deviation (SD) or standard error (SE). The Shapiro-Wilk test was used to estimate parametric and non-parametric data. A Spearman or Pearson correlation was used to calculate the correlation between two independent variables. A paired or unpaired samples t-test was used to calculate the difference between two related groups and two independent groups, respectively. One-way analysis of variance (ANOVA) was used to calculate the significant difference between three or more independent groups. Repeated measures ANOVA was used to calculate the significant difference between three or more related groups. A Dunnett's multiple comparison test was used to compare the differences of the groups against the control. A Tukey's multiple comparisons test was used to compare all groups to each other for parametric data. A Dunn's multiple comparisons test was used to compare all groups to each other for non-parametric data. All p-values <0.05 were considered to be statistically significant. Statistical analysis was carried out using IBM SPSS Statistics 24 or GraphPad Prism 7.04.

3 The Effects of Acute Hyperglycaemia on Platelet Function *In Vitro*

3.1 Introduction

The clinical diagnosis of diabetes is based on HbA1c over 48 mmol/mol, and large-scale studies have linked high blood glucose with cardiovascular disease in type 2 diabetic patients (Stratton et al., 2000; Coutinho et al., 1999 and Turnbull et al., 2009). To prevent cardiovascular events from occurring, diabetic patients are prescribed antiplatelet medication such as aspirin. Despite this, clinical studies have shown that the response to antiplatelet drugs is weaker in diabetic patients compared to non-diabetic people with vascular complications (Angiolillo, 2009).

A number of *ex vivo* and *in vitro* studies have investigated the effect of hyperglycaemia on platelet reactivity, however, the data is contradictory (see section 1.7). Consequently, the literature does not confirm that hyperglycaemia is causing platelet hyperreactivity in diabetes. To further investigate the effect of high glucose on platelet reactivity, this study used *in vitro* techniques to measure platelet activation and aggregation in the presence of increasing glucose concentrations. The study also investigated the effect of high glucose on NO and PGI_2-mediated platelet inhibition.

The aims of this chapter were to determine whether acute elevations in glucose levels have a direct effect on platelet function. Alterations in platelet size and activation state were analysed in resting platelets from healthy donors following incubation with increasing concentration of glucose or mannitol. Platelet reactivity to standard physiological platelet agonists were also assessed, to determine whether acute hyperglycaemia has the potential to directly promote a

prothrombotic platelet phenotype in the circulation. The effects of hyperglycaemia on the platelet inhibitory pathways mediated by NO and PGI_2 were also investigated.

3.2 Results

3.2.1 The Effect of Acute Glucose on Platelet Size

Much of the work so far, investigating hyperglycaemia and platelet function reported in the literature, has looked at the relationship between MPV and high glucose *ex vivo*. To establish whether acute glycaemia in the circulation can directly influence platelet size, an *in vitro* method was employed.

For the *in vitro* method, the platelet count, MPV, PDW and P-LCR were measured in whole blood from fasted healthy participants, incubated with increasing glucose concentrations at 37°C for 1 hour. Mannitol was used as a metabolically-inactive control, to investigate the effects of platelet swelling.

The mean $\pm$ SD of fasting glucose for the healthy subjects before testing was 4.62 $\pm$ 0.56 mmol/L (n=10). This mean is within the 3.5-5.5 mmol/L range, representative of normal fasting glucose for healthy people (Tuch et al., 2000). The platelet parameters platelet count, MPV, P-LCR and P-LCR for the ten healthy subjects were initially assessed to ensure that they were in the normal range (Table 3.1.).

Table 3.1: Baseline mean values for platelet parameters in healthy subjects

Platelet parameter	*Mean ± SD (n=10)*	*Normal Range*
Platelet Count (x10^9/L)	243.7 ± 74.64	150 – 450[≠]
Mean platelet volume (fL)	10.54 ± 1.05	7.0 – 10.5[≠]
Platelet distribution width (fL)	12.73 ± 2.38	10.0 - 17.9[$]
Platelet-large cell ratio (%)	29.64 ± 8.69	15.0 – 35.0[≈]

Normal ranges taken from the following sources: [≠]Giles, 1981; [$] Farias et al., 2010; [≈]Budak et al., 2016.

The unit of measurement for MPV (femtolitres (fL)) is considerably small so any significant differences may not be shown, therefore the data was normalised to represent the fold change against the control. A repeated measures one-way ANOVA showed no significant difference between the mean platelet count for the four glucose concentrations (Table 3.2). There was a statistically significant difference between the mean MPV values for all four glucose concentrations (p=<0.0001) (Table 3.2 and Figure 3.1), together with PDW (p=0.004) and P-LCR (p=<0.0001) (Table 3.2). A Dunnett's multiple comparison test showed that the 5, 15 and 30 mmol/L added glucose significantly increased the MPV when compared to the control (p=0.025, p=<0.0001, p=0.0001 respectively) (Figure 2.1). In contrast, the osmotic control, mannitol, had no significant effect on the MPV (p=0.491) (Table 3.3 and Figure 3.2). The 30 mmol/L added glucose also had a significant effect on PDW when compared to no added glucose (p=0.008). For P-LCR, 5, 15 and 30 mmol/L were significantly increased compared to the control (p=0.0125, p=0.0001 and p=0.0003, respectively). Mannitol had no significant effect on PDW (p=0.118), however there was a small but significant reduction in P-LCR for 15 mmol/L compared to the control (p=0.021).

Table 3.2: Fold change for platelet parameters in blood with increasing glucose concentrations

Added glucose (mmol/L)	*0.0*	*5.0*	*15.0*	*30.0*	*p-value*
Platelet count (x10^9/L)	1.0 ± 0.0	0.99 ± 0.02	1.01 ± 0.03	0.98 ± 0.02	0.467
MPV (fL)	1.0 ± 0.0	1.02 ± 0.005	1.03 ± 0.004	1.06 ± 0.008	**<0.0001**
PDW (fL)	1.0 ± 0.0	1.05 ± 0.03	1.07 ± 0.03	1.10 ± 0.03	**0.004**
P-LCR (%)	1.0 ± 0.0	1.05 ± 0.01	1.11 ± 0.02	1.20 ± 0.03	**<0.0001**

Fold change for platelet parameters in healthy blood with added glucose (5-30 mmol/L) when compared to the non-treated blood (0 mmol/L added glucose). A repeated measures one-way ANOVA was used to calculate the p value. Values presented as mean ± SEM (n=10).

Table 3.3: Fold change for platelet parameters in blood with increasing mannitol concentrations

Added mannitol (mmol/L)	*0.0*	*5.0*	*15.0*	*30.0*	*p-value*
Platelet count (x10^9/L)	1.0 ± 0.0	1.05 ± 0.02	0.99 ± 0.02	1.02 ± 0.02	0.061
MPV (fL)	1.0 ± 0.0	1.00 ± 0.007	0.99 ± 0.004	1.00 ± 0.006	0.491
PDW (fL)	1.0 ± 0.0	0.98 ± 0.02	0.98± 0.006	0.98 ± 0.009	0.118
P-LCR (%)	1.0 ± 0.0	1.00 ± 0.02	0.97 ± 0.01	0.97 ± 0.01	**0.028**

Fold change for platelet parameters in healthy blood with mannitol (5-30 mmol/L) when compared to the non-treated blood (0 mmol/L added glucose). A repeated measures one-way ANOVA was used to calculate the p value. Values given as mean ± SEM (n=10).

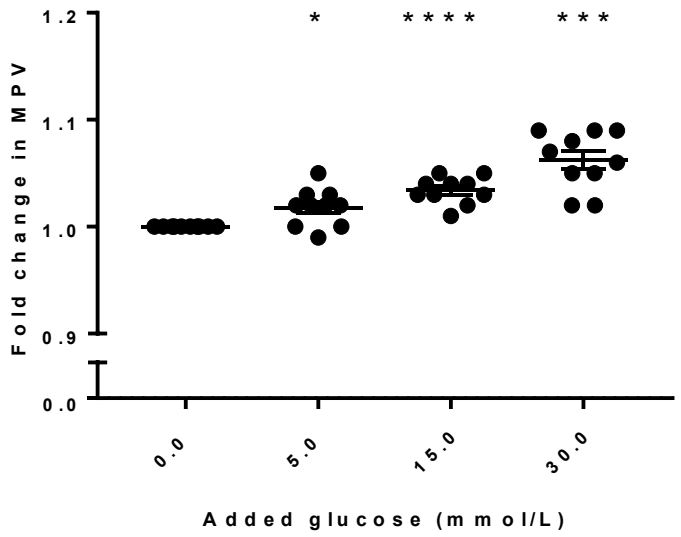

Figure 3.1: The effect of added glucose on MPV in healthy blood
Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and the MPV measured. Values given as mean ± SEM (n=10). Data analysed using a Dunnett's multiple comparison test (*p=<0.0001 and *p=0.0001).

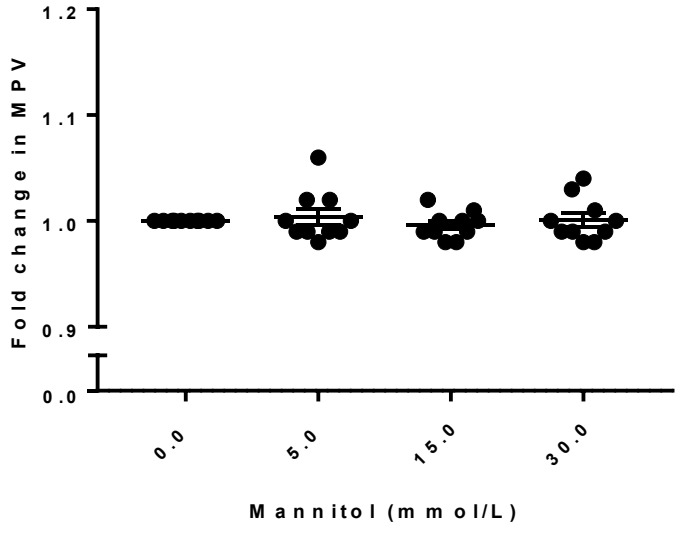

Figure 3.2: The effect of mannitol on MPV in healthy blood
Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and the MPV measured. Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (1.9, 17.1) = 0.728, p=0.491).

3.2.2 The Effect of Acute Glucose on Platelet Activation

In the *in vitro* study investigating the effect of high glucose on platelet size (section 3.2.1), it was identified that MPV is increased in the presence of glucose but not mannitol, demonstrating a direct effect on peripheral platelets. To investigate whether the increased size is also accompanied by an increase in surface activation markers, the effects of hyperglycaemia on platelet P-selectin (CD62P) surface expression and $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) was assessed on resting platelets in whole blood using flow cytometry. P-selectin is an adhesion molecule stored in platelet α-granules and translocated to the platelet surface upon α-granule secretion. PAC-1 is an antibody, which specifically binds to activated $\alpha_{IIb}\beta_3$ on the platelet surface, and is an indication of platelet aggregation.

To prevent results being affected by patient variability, the data was normalised by calculating the fold change of the control. The mean $\pm$ SD baseline glucose concentration for the participants was 4.72 $\pm$ 0.42 (n=10). A repeated measures one-way ANOVA showed a trend towards an increase in P-selectin surface expression with increased glucose concentrations (p=0.067) (Figure 3.3). A significant increase in PAC-1 binding was shown with increased glucose (p=0.024) (Figure 3.4). A Dunnett's multiple comparison test showed that 15 mmol/L added glucose was significantly higher compared to the 0 mmol/L glucose control (p=0.032). 5 mmol/L glucose was not significantly different to the control (p=0.172), but 30 mmol/L glucose was very close to significance (p=0.051).

To investigate the effect of hyperglycaemia on activated platelets, CD62P surface expression and PAC-1 binding was assessed in platelets stimulated with ADP in whole blood flow cytometry. A repeated measures one-way ANOVA showed no significant effect in P-selectin surface expression increasing glucose concentrations (p=0.389) (Figure 3.5). However, a repeated measures one-way ANOVA showed a significant decrease in PAC-1 binding (p=0.010) (Figure 3.6). A Dunnett's multiple comparison test showed that 30 mmol/L added glucose was significantly decreased compared to the 0 mmol/L glucose control (p=0.025). 5 mmol/L and 15 mmol/L added glucose were not significantly different to the control (p=0.312 and p=0.389, respectively).

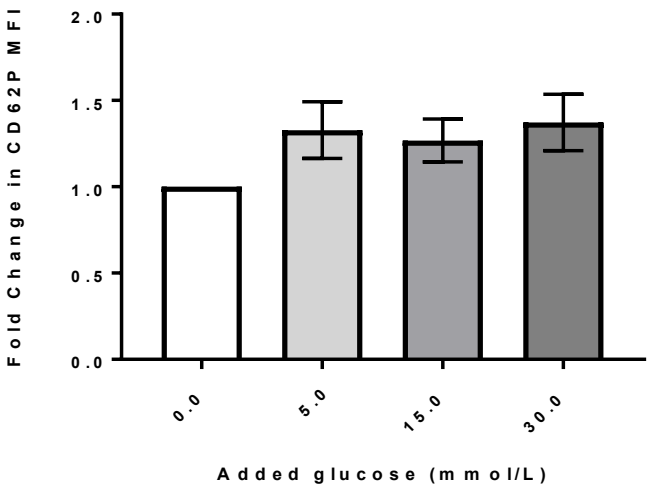

Figure 3.3: The effect of glucose on α-granule secretion in rested platelets
Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30mmol/L) for 1h and surface expression of P-selectin measured using flow cytometry. Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (2.488, 22.39) = 2.874, p=0.0674).

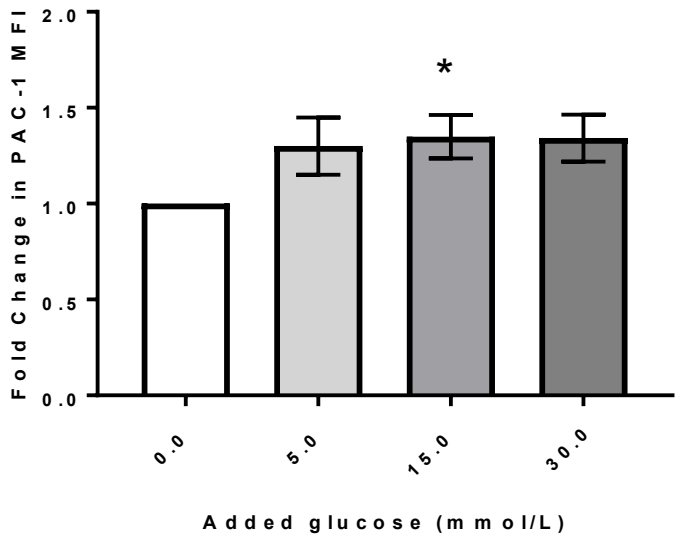

Figure 3.4: The effect of glucose on PAC-1 binding in resting platelets
Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30mmol/L) for 1h and PAC-1 binding measured using flow cytometry. Values given as mean ± SEM (n=10). Data analysed using a Dunnett's multiple comparison test (*p=0.032).

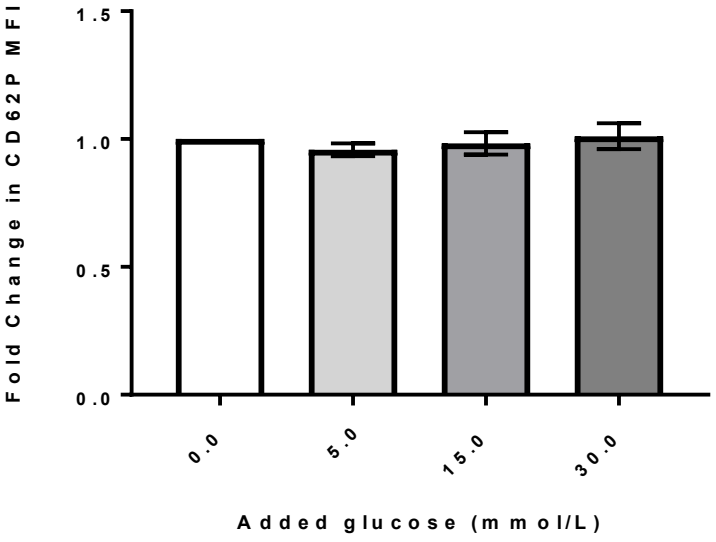

Figure 3.5: The effect of glucose on α-granule secretion in ADP-activated platelets
Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and surface expression of P-selectin measured using flow cytometry. Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (1.278, 11.5) = 4.604, p=0.389).

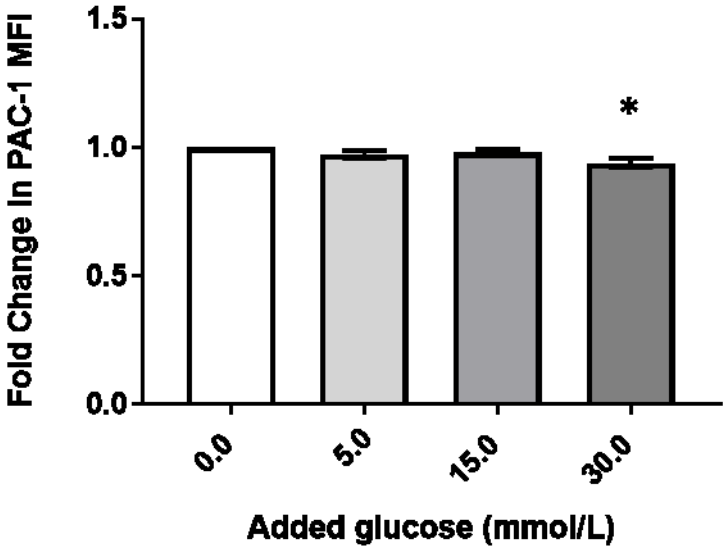

Figure 3.6: The effect of glucose on PAC-1 binding in ADP-activated platelets

Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and PAC-1 binding measured using flow cytometry. Values given as mean ± SEM (n=10). Data analysed using a Dunnett's multiple comparison test (*p=0.025).

To identify whether platelets are indirectly affected by high glucose, through reduced sensitivity to platelet inhibitors, the efficacy of the nitric oxide donor SNP and PGE_1 was assessed in hyperglycaemic conditions and platelet activation measured by flow cytometry.

The mean baseline glucose for the participants was 4.76 ± 0.58 mmol/L (n=5). Data was represented as the % inhibition of basal conditions and this was calculated as described in section 2.5.1. Interestingly, the inhibition of α-granule secretion evoked by SNP (100 μmol/L) was significantly enhanced in the presence of 30 mmol/L glucose (p=0.036). No difference was observed for PAC-1 binding (p=0.202) (see figure 3.7). Glucose had no effect on CD62P surface

expression or PAC-1 binding in the presence of PGE_1 (0.25 µmol/L) (p=0.536 and p=0.353, respectively) (see figure 3.8).

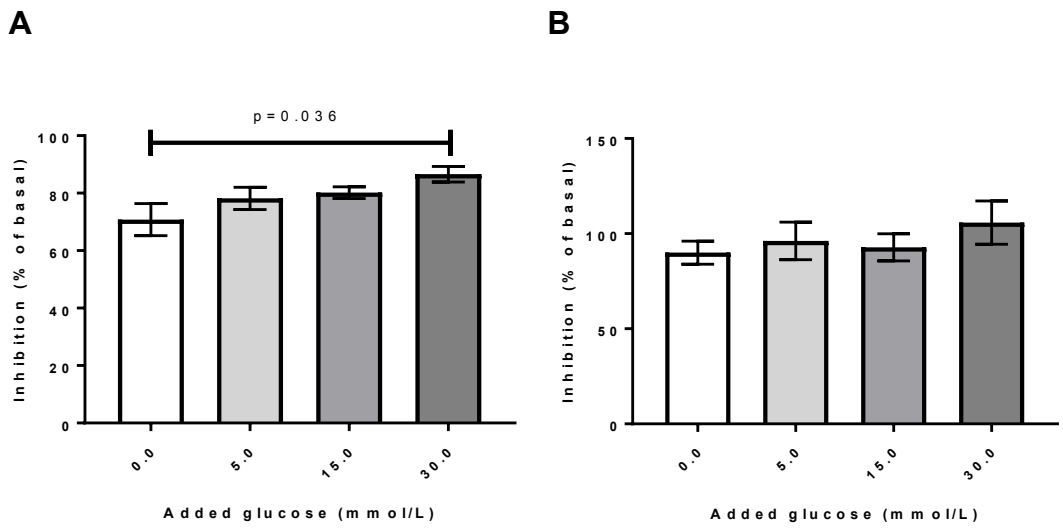

Figure 3.7: The effect of glucose and SNP-mediated inhibition on platelet activation

Figure A: Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) and CD62P surface expression was measured in platelets stimulated with ADP (2.5 µmol/L) with and without SNP (100 µmol/L). Values given as mean ± SEM (n=5). Data is represented as the % inhibition (of basal). Data calculated using a repeated measures one-way ANOVA (F (1.844, 7.376) = 5.49, p = 0.036). **Figure B:** Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) and PAC-1 binding was measured in platelets stimulated with ADP (2.5 µmol/L) with and without SNP (100 µmol/L). Values given as mean ± SEM (n=5). Data was analysed as % of basal inhibition, using a repeated measures one-way ANOVA (F (1.237, 4.949) = 2.206, p = 0.202).

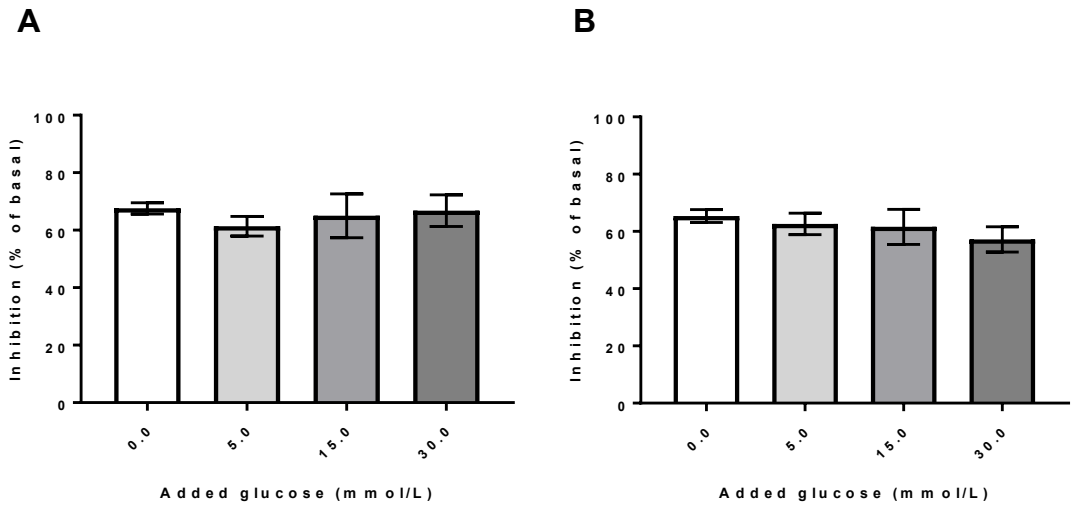

Figure 3.8: The effects of glucose and PGE_1-mediated inhibition on platelet activation

Figure A: Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) and CD62P surface expression was measured in platelets stimulated with ADP (2.5 µmol/L) with and without PGE_1 (0.25 µmol/L). Values given as mean ± SEM (n=5). Data was analysed as % of basal inhibition, using a repeated measures one-way ANOVA (F (1.473, 5.893) = 0.589, p = 0.536). **Figure B:** Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) and PAC-1 binding was measured in platelets stimulated with ADP (2.5 µmol/L) with and without PGE_1 (100 µmol/L. Values given as mean ± SEM (n=5). Data was analysed as % of basal inhibition, using a repeated measures one-way ANOVA (F (1.883, 7.531) = 1.187, p = 0.353).

3.2.3 The Effect of Glucose on Platelet Aggregation

To investigate whether acute hyperglycaemia directly modulates platelet reactivity, PRP from healthy subjects was incubated with increasing concentrations of glucose and stimulated with submaximal concentrations of physiological platelet agonists collagen or ADP.

The mean ± SD of fasting glucose for the healthy subjects before testing was 4.48 mmol/L ± 0.36 mmol/L (n=5). This mean is within the 3.5-5.5 mmol/L range, representative of normal fasting glucose for healthy people (Tuch et al., 2000).

PRP was incubated with glucose for 1 hour at room temperature. Platelet aggregation was measured in response to submaximal concentrations of ADP (2.5 µmol/L) and collagen (1.0 µg/ml), elucidated from a dose response (Appendix 9.1). LTA demonstrated that glucose did not significantly alter ADP- or collagen-stimulated platelet aggregation ($p=0.395$ and $p=0.431$, respectively) (Figure 3.9 and Figure 3.10).

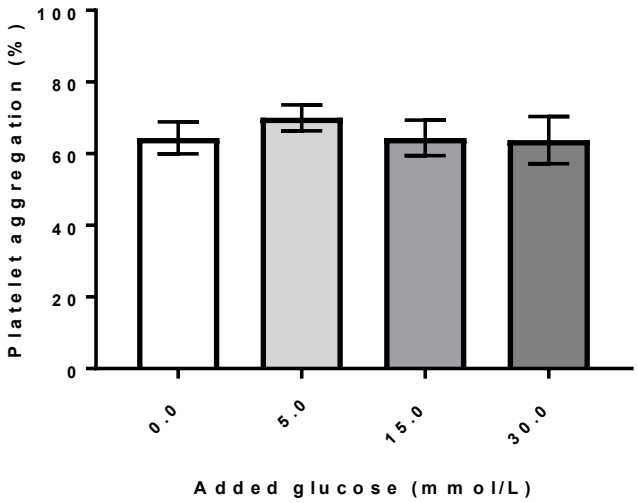

Figure 3.9: The effect of glucose on ADP-activated platelet aggregation in PRP

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and platelet aggregation was stimulated with ADP (2.5 µmol/L). Values given as mean ± SEM (n=5). Data analysed using a repeated measures one-way ANOVA (F (1.455, 5.778) = 0.995, p=0.395).

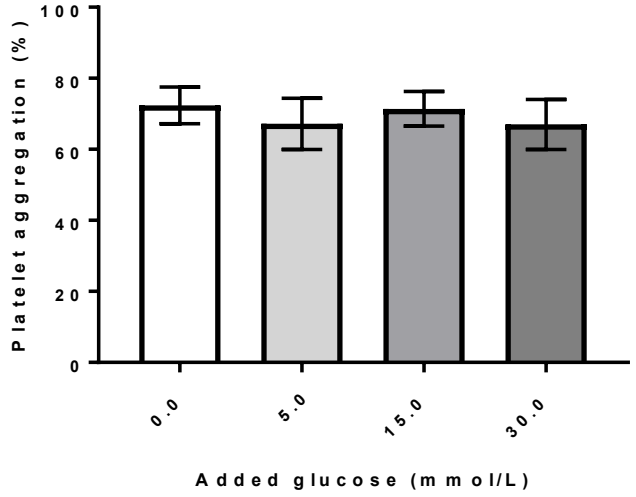

Figure 3.10: The effect of glucose on collagen-induced platelet aggregation in PRP

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h and platelet aggregation was stimulated with collagen (1.0 µg/ml). Values given as mean ± SEM (n=5). Data analysed using a repeated measures one-way ANOVA (F (1.051, 4.203) = 0.781, p=0.431).

The literature has shown that research teams used different conditions to measure platelet aggregation in the presence of glucose (section 1.7.3). Thus, PRP was incubated with glucose at 37^{o}C for 1 hour to investigate whether a physiologically relevant temperature would give a significant effect on platelet aggregation.

The mean $\pm$ SD of the fasting glucose concentration for the healthy subjects before experimentation was 4.52 mmol/L $\pm$ 0.16 mmol/L (n=10), within the range for normal fasting glucose levels.

Contrary to the results obtained at room temperature, there was a surprising dose dependent reduction in platelet aggregation shown with ADP (2.5 µmol/L) activation for 15 and 30 mmol/L glucose (see Figure 3.11 and Figure 3.12) (Dunnett's, p=0.036 and p=0.007, respectively). Conversely, glucose concentration did not have a significant effect on aggregation stimulated with collagen (1.0 µg/ml) (p=0.435) (Figure 3.13).

To further investigate the effect of glucose on platelet function, luminescence aggregometry was undertaken in six healthy participants, to measure the release of ATP in PRP stimulated with ADP (2.5 µmol/L) and collagen (1.0 µg/ml). Although there was a dose-dependent reduction in ATP release for ADP-activated PRP, a repeated measures one-way ANOVA demonstrated that there was no significant difference (p=0.152) (Figure 3.14). No significant difference was shown with collagen (p=0.461) (Figure 3.15).

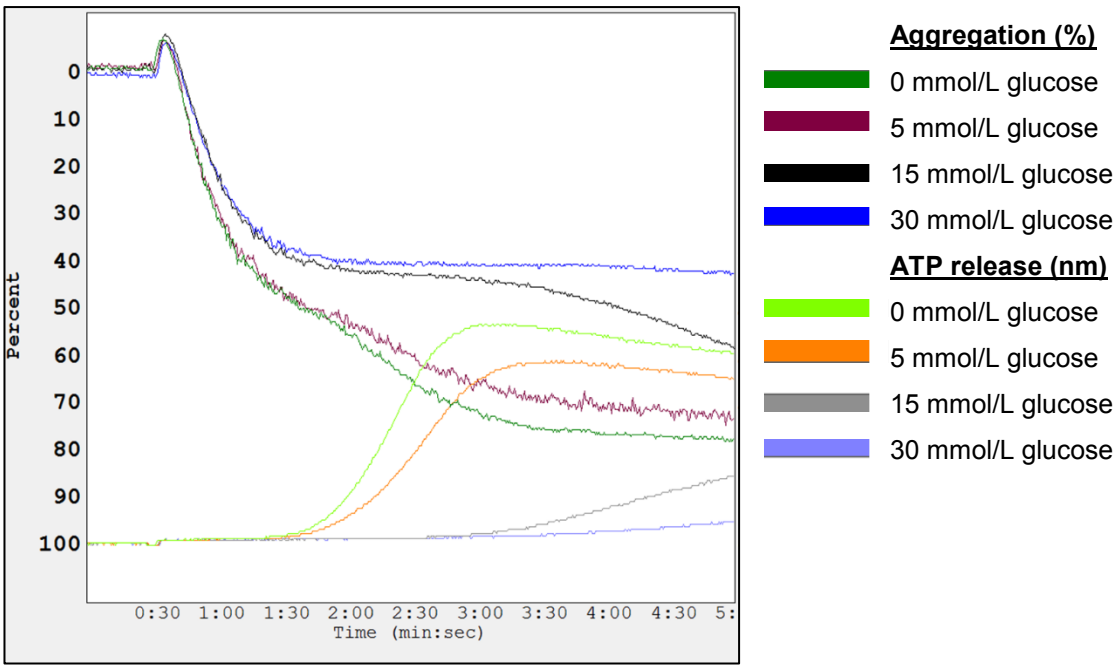

Figure 3.11: Platelet aggregation and ATP release induced with ADP

An example of platelet aggregation (%) and ATP release (nm) from one fasted healthy participant. The image shows that both the ATP release and platelet aggregation was reduced as the glucose concentration was increased. In the legend the colours represent the glucose concentration (mmol/L) for platelet aggregation (%) and ATP release (nm).

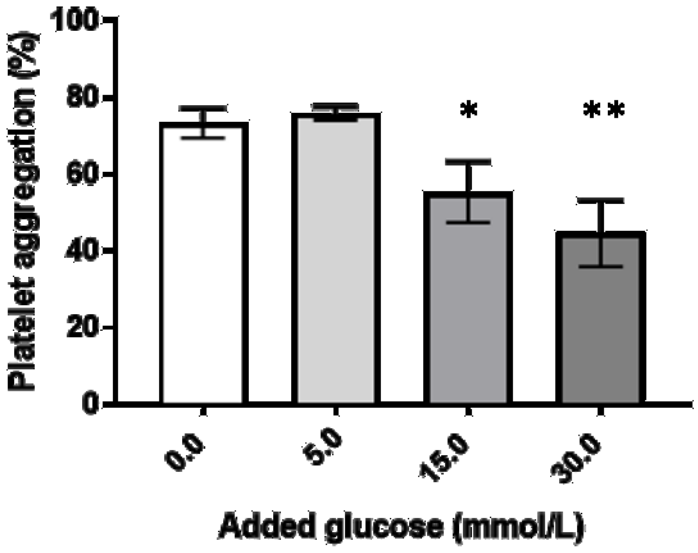

Figure 3.12: The effect of glucose on ADP-induced platelet aggregation in PRP

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and platelet aggregation was stimulated with ADP (2.5 µmol/L). Values given as mean ± SEM (n=10). Data analysed using a Dunnett's multiple comparison test (*p=0.036 and *p=0.007).

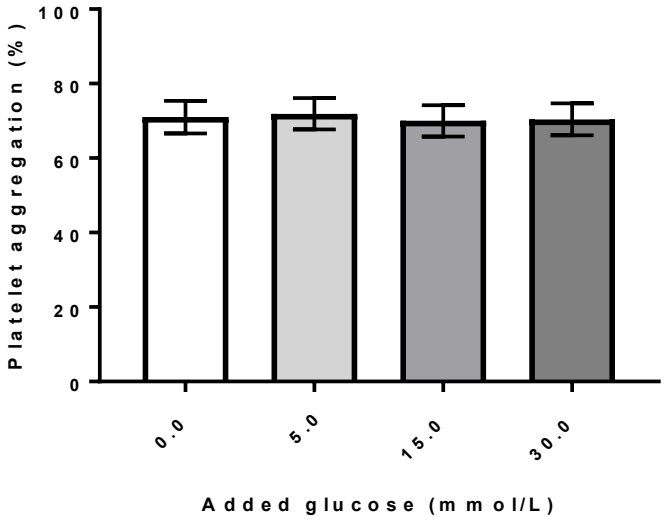

Figure 3.13: The effect of glucose on collagen-induced platelet aggregation in PRP

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and platelet aggregation was stimulated with collagen (1.0 µg/ml). Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (2.632, 23.69) = 0.922, p = 0.435).

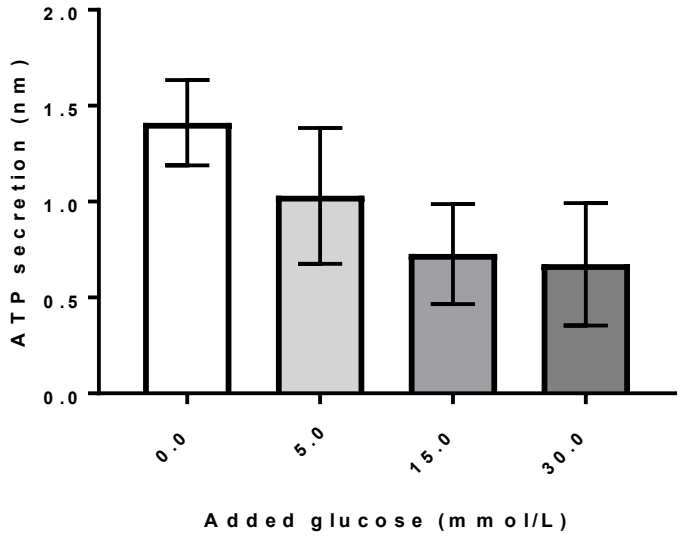

Figure 3.14: Effect of glucose on ATP secretion in PRP activated with ADP

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and ATP secretion was measured in platelets stimulated with ADP (2.5 µmol/L). Values given as mean ± SEM (n=6). Data analysed using a repeated measures one-way ANOVA F (1.459, 7.297) = 2.529, p=0.152).

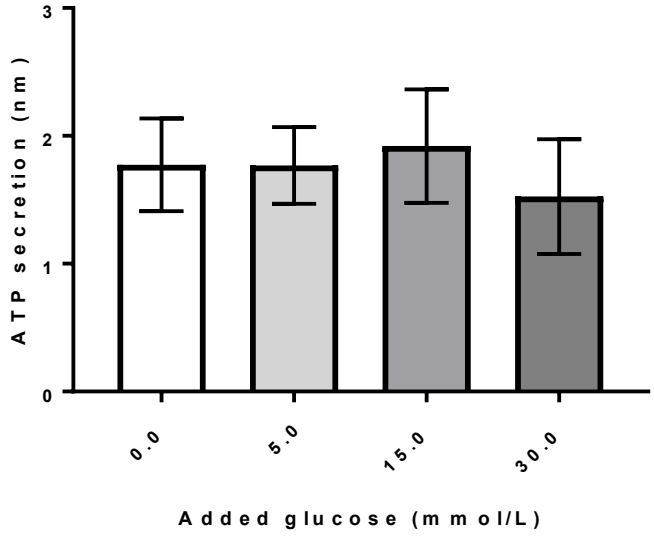

Figure 3.15: Effect of glucose on ATP secretion in PRP induced with collagen

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and ATP secretion was measured in platelets stimulated with collagen (1.0 µg/ml). Values given as mean ± SEM (n=6). Data analysed using a repeated measures one-way ANOVA (F (1.786, 8.932) = 0.812, p=0.461).

To investigate the effect of glucose in the presence of other blood components, platelet aggregation was measured in whole blood. The blood was stimulated with ADP (2.5 µmol/L) and collagen (1.0 µg/ml) to induce platelet aggregation.

The mean fasting glucose concentration of the healthy subjects before experimentation was 5.29 ± 0.22 (n=10). The results show no significant difference between the glucose concentrations for whole blood stimulated with ADP or collagen (p=0.248 and p=0.199, respectively) (Figure 3.16 and Figure 3.17).

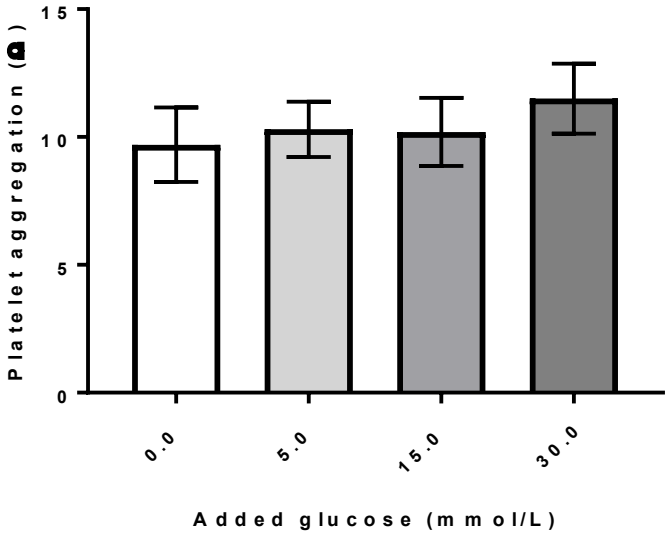

Figure 3.16: The effect of glucose on ADP-induced platelet aggregation in whole blood

Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and maximal aggregation was measured in platelets stimulated with ADP (2.5 µmol/L). Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (2.261, 20.35) = 1.494, p=0.248).

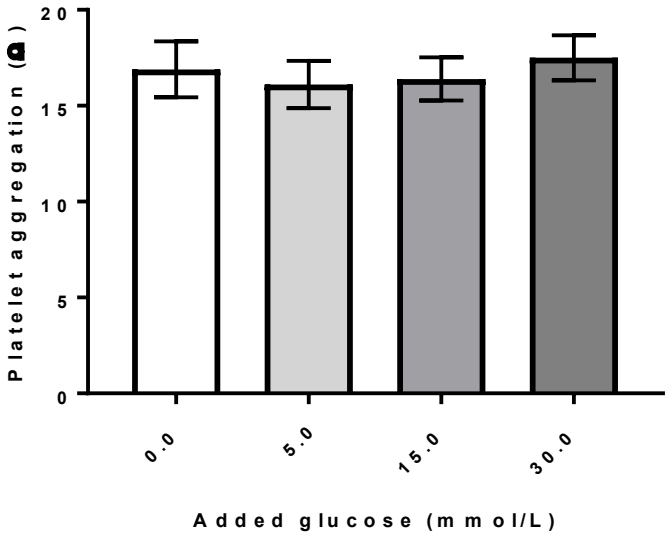

Figure 3.17: The effect of glucose on collagen-induced platelet aggregation in whole blood

Whole blood from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and maximal aggregation was measured in platelets stimulated with collagen (1.0 µg/ml). Values given as mean ± SEM (n=10). Data analysed using a repeated measures one-way ANOVA (F (1.58, 14.22) = 1.829, p=0.199).

Previous results from this study showed that high glucose did not directly affect platelet hyperreactivity. To further investigate whether platelets are indirectly affected by high glucose, through reduced sensitivity to platelet inhibitors, platelet aggregation was measured in the presence of SNP or PGE_1 under hyperglycaemic conditions.

To determine a suitable concentration of SNP and PGE_1, a dose response was performed, and the concentration that almost abolished ADP-induced platelet aggregation was selected for SNP (100 µmol/L) and PGE_1 (0.25 µmol/L) (Appendix 9.1).

Acute elevation in glucose levels had no significant effect on the inhibition of ADP-mediated aggregation, evoked by SNP (100 µmol/L) (p=0.645) (Figure 3.18). Similarly, the inhibition of platelet aggregation achieved with PGE_1 (0.25 µmol/L) (p=0.212) (Figure 3.19), was not significantly altered by elevated glucose levels.

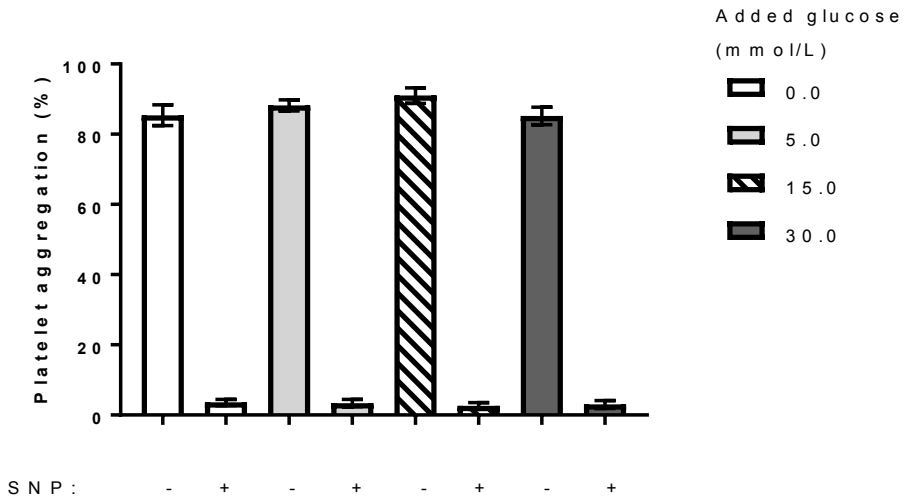

Figure 3.18: The effect of glucose and SNP-mediated inhibition on platelet aggregation

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and maximal aggregation was measured in platelets stimulated with ADP (2.5 µmol/L) with and without SNP. Values given as mean ± SEM (n=5). Data analysed using a repeated measures one-way ANOVA without SNP (F (1.87, 7.481) = 1.552, p=0.272) and with SNP (100 µM) (F (1.797, 7.188 = 0.432, p=0.645).

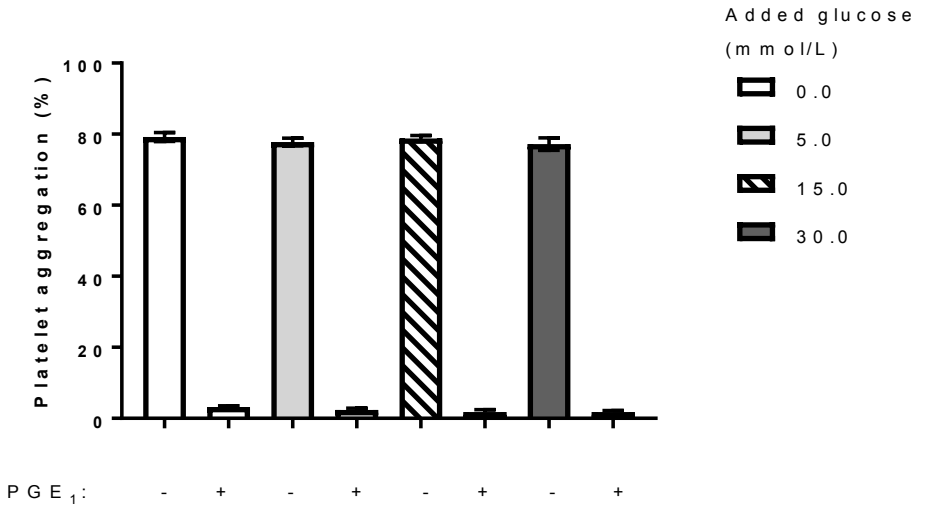

Figure 3.19: The effect of glucose and PGE1-mediated inhibition on platelet aggregation

PRP from healthy fasted donors was supplemented with increasing concentrations of glucose (0-30 mmol/L) for 1h at 37ºC and maximal aggregation was measured in platelets stimulated with ADP (2.5 µmol/L) with and without PGE_1. Values given as mean $\pm$ SEM (n=5). Data analysed using a repeated measures one-way ANOVA without PGE_1 (F (1.392, 5.567) = 0.508, p = 0.564) and with PGE_1 (0.25 µmol/L) (F (1.988, 7.95) = 1.899, p=0.212).

3.2 Discussion

Hyperglycaemia is recognised as the main cause of vascular complications in diabetes. Despite this, both *ex vivo* and *in vitro* studies investigating the effect of high glucose on platelet hyperreactivity report contradictory conclusions.

Larger platelets with MPV tend to be more reactive and release more prothrombotic factors such as TXA_2 (Shimodaira et al., 2014). Therefore, MPV is considered to be an independent risk factor for myocardial infarction and stroke, and it is emerging as a clinical biomarker for cardiovascular disease. The majority of *ex vivo* studies investigating the relationship between MPV and HbA1c in type 2 diabetes identified a positive correlation (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder et al, 2014; Ulutas et al., 2014). However, similar studies disagreed with this data (Hekimsoy et al., 2004 and Pananas et al., 2004). Additionally, data from *ex vivo* studies investigating the relationship between MPV and FBG in type 2 diabetic patients report both a strong relationship (Kodiatte et al., 2012; Ozder et al., 2014; Ulutas et al., 2014) and no relationship (Demirtunc et al., 2009; Hekimsoy et al., 2004). Furthermore, no study has investigated the relationship between MPV and high glucose using *in vitro* techniques. Therefore, it is unclear whether any alterations in platelet size were through increased thrombopoeisis and release of larger immature platelets, or whether glucose has a direct effect on platelets in the circulation.

Using *in vitro* techniques, this study showed that a significant increase in MPV with increasing glucose concentrations ($p=<0.0001$). This is possibly due to the uptake of glucose by the GLUT transporters which may lead to an increase in

water diffusion into the cell causing cell swelling, as shown by Lösche et al. (1989) in erythrocytes. This suggests that hyperglycaemia enhances platelet size directly in the circulation. It doesn't, however, rule out that glucose may influence megakaryocytes and thrombopoiesis as well.

It has been suggested that circulating platelets can be primed so that they have an increased response to stimuli. For example, Massberg et al. (2002) reported that mice with atherosclerosis had circulating platelets that expressed surface P-selectin, which potentiated the inflammatory response and contributed to atherogenesis. To assess whether the increase in platelet size, stimulated by acute hyperglycaemia, was accompanied by an elevation in activation markers, surface levels of P-selectin and activated $\alpha_{IIb}\beta_3$ were measured. In resting platelets, there was no significant difference in α-granule secretion. There was a small but significant increase in PAC-1 binding observed, and this may represent an increase in activation of the $\alpha_{IIb}\beta_3$ receptors present, but not an upregulation in $\alpha_{IIb}\beta_3$ which occurs following α-granule secretion. A recent study investigating the effect of compression force on circulating platelets from a streptozotocin murine model of diabetes, also reported elevated activation of $\alpha_{IIb}\beta_3$ (Ju et al., 2018). Additionally, the team identified that >95% of the platelets did not have P-selectin expression, supporting the notion that hyperglycaemia does not increase surface P-selectin expression. Also consistent with our study, they showed no difference in agonist-induced platelet activation between control and diabetic platelets, indicating that hyperglycaemia does not directly enhance platelet reactivity. Ju et al., (2018) did however demonstrate a distinct pressure dependent mechanism, induced by hyperglycaemia, which enhanced the affinity

of $\alpha_{IIb}\beta_3$ for fibrinogen, through a PI3K–dependent mechanism. It is possible that this could be a consequence of the cell swelling effects observed in our study, since cell swelling has been shown to activate the PI3K/AKT signalling axis in other cell types (Webster et al., 2000).

No significant increase in platelet aggregation was observed in either PRP or whole blood experiments. However, there was a significant reduction in ADP-activated platelet aggregation in the presence of 15 mmol/L and 30 mmol/L glucose at 37^{o}C. Furthermore, PAC-1 binding was significantly reduced in ADP-activated platelets incubated with 30 mmol/L glucose. The high glucose could be triggering platelet apoptosis, yet the results in section 3.2.1 demonstrate that the platelet count remains the same in high glucose compared to low glucose concentrations. A possible explanation is that the high glucose increases ecto-nucleotidases, which, in turn, increases the hydrolysis of ATP/ADP to AMP and adenosine (Lunkes et al., 2008). This would reduce the level of ADP that would otherwise trigger the activation of circulating platelets.

Acute glucose does not reduce the inhibitory effect of SNP and PGE_1. This supports other *in vitro* studies that have reported no influence of inhibitory activity on platelet activation under high glucose conditions (Sudic et al., 2006; Russo et al. 2012). Although it has been demonstrated that glucose impairs the ability for aspirin to activate the NO/cGMP pathway (Russo et al. 2012), suggesting that glucose alters the efficacy of antiplatelet therapy rather than altering NO activity per se. The inhibitory effect was significantly increased for CD62P surface

expression in SNP-treated platelets. This, again, supports the idea that high glucose increases ecto-nucleotidases, further increasing platelet inhibition.

In conclusion, whilst the data demonstrates that acute hyperglycaemia directly enhances platelet size and may lead to low-level $\alpha_{IIb}\beta_3$ activation, there is no evidence to suggest that it has any effect on platelet reactivity and responsiveness to agonist stimulation. Furthermore, the lack of platelet hyperreactivity in hyperglycaemic conditions observed in this study is in contrast with several published studies (Keating et al., 2003; De la Cruz et al., 2004; Tang et al., 2011) and the experimental conditions may contribute to the outcomes.

4 Identification of Biochemical Parameters Associated with Platelet Reactivity in Type 2 Diabetic Patients

4.1 Introduction

In the previous chapter, *in vitro* studies indicated that acute hyperglycaemia does not enhance platelet reactivity directly through potentiating agonist responses or indirectly through reducing platelet sensitivity to inhibitor signalling pathways.

Since the viability of platelets *in vitro* is limited to a few hours, the chronic effects of glucose on platelet function cannot be investigated by glucose supplementation. *In vitro* studies also do not allow for altered platelet function through effects on the parent megakaryocyte to be interrogated. In order to examine the effects of hyperglycaemia on platelets over a longer period, *ex vivo* experiments were undertaken using blood from type 2 diabetic patients.

As well as hyperglycaemia, type 2 diabetes is associated with alterations in the blood milieu caused by dyslipidaemia and oxidative stress (Kakouros et al., 2011). Thus, correlations between platelet reactivity and lipoprotein levels (LDL-C, HDL-C, triglycerides and ox-LDL) was also investigated. Clinical characteristics were also incorporated into the correlations to determine whether platelet reactivity is associated with age, BMI or duration of diabetes. Measurements of platelet reactivity include platelet indices (e.g. platelet count, MPV), IPF (as a measurement of platelet production), platelet activation and aggregation.

4.2 Results

4.2.1 Baseline Characteristics for the Type 2 Diabetic Cohort

A total of forty subjects diagnosed with type 2 diabetes were recruited for this study. A summary of the baseline characteristics for the type 2 diabetic patients are given in Table 4.1. There were twenty female diabetics and twenty male diabetics in the study. The mean age of the diabetic population was 58.6 ± 9.1 years. The mean duration of diabetes was 11.9 ± 5.8 years. The mean BMI was 32.3 + 5.8 kg/m^2, with twenty-four (60.0%) of the forty patients with a BMI over 30 kg/m^2 and categorised as obese (World Health Organisation, 2017). Only four (10.0%) of the forty diabetic subjects was a current smoker and thirteen (32.5%) were previous smokers.

Out of the forty diabetic subjects, twenty-three (57.5%) were injecting insulin, and thirty-two (80.0%) were taking other anti-hyperglycaemic medication such as Metformin, sulfonylureas, SGL-2 inhibitors and DPP-4 inhibitors. Twenty-six (65.0%) were taking antihypertensive medication and thirty-five (87.5%) were taking lipid lowering medication, with thirty-two (80.0%) taking statins.

The mean FBG was 9.2 ± 3.6 mmol/L. The mean HbA1c level was 66.6 ± 19.1 mmol/mol. The mean total cholesterol was 4.7 ± 1.3. The mean HDL-C was 1.3 ± 0.5 mmol/L. The mean LDL-C was 2.4 ± 1.0 mmol/L. The mean triglyceride level was 2.1 ± 1.1 mmol/L. The serum creatinine gives an indication of kidney function and the range (min-max) for females was 51-123 µmol/L and for males 63-190 µmol/L. Normal reference ranges are between 45-84 µmol/L for females

and 59-104 μmol/L for males (Central Manchester University Hospitals NHS Foundation Trust, 2018). The mean values for platelet count and MPV, PDW and P-LCR are given in table 4.2.

Table 4.1: Baseline characteristics for the type 2 diabetic cohort

	n=40
M/F (*n*)	20/20
Age (yr), mean ± SD	58.6 ± 9.1
Duration of diabetes (yr), mean ± SD	11.9 + 5.8[$]
BMI (kg/m^2), mean ± SD	32.3 + 5.8
Current smoker (%)	10.0
Previous smoker (%)	32.5
Medication Profile (%)	
Insulin	57.5
Other anti-hyperglycaemic medication	80.0
Antihypertensive agents	65.0
Lipid lowering drugs (incl. statins)	87.5
History of (%)	
Peripheral neuropathy	17.5
Retinopathy	17.5
Acute kidney injury	7.5
Hypertension	70.0
Laboratory variables	
Fasting blood glucose (mmol/L), mean ± SD	9.2 ± 3.6
HbA1c (mmol/mol), mean ± SD	66.6 ± 19.1
Total Cholesterol (mmol/L), mean ± SD	4.7 ± 1.3
LDL-C (mmol/L), mean ± SD	2.4 ± 1.0
HDL-C (mmol/L), mean ± SD	1.3 ± 0.5
Triglycerides (mmol/L), mean ± SD	2.1 ± 1.1
Serum creatinine (µmol/L), range (min-max)	Female: 51-123, Male: 63-190

[$] denotes missing data for 1 patient (n=39)

4.2.2 Platelet Indices

Elevated MPV has been reported to be associated with the risk of myocardial infarction (Martin et al., 1991) and hyperglycaemia (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder et al, 2014; Ulutas et al., 2014). The measurement of MPV is a cheap, effective test that may be used to predict cardiovascular risk in type 2 diabetic patients. The aim of this study was to investigate the relationship between platelet count and platelet size in type 2 diabetic patients.

Table 4.2: Platelet parameters for the type 2 diabetic cohort

Platelet parameter	*Mean ± SD (n=40)*
Platelet count (x10^9/L)	250.73 ± 54.93
MPV (fL)	10.75 ± 1.08
PDW (fL)	13.73 ± 2.57
P-LCR (%)	33.23 ± 8.95

Platelet count for all patients fell within the normal range and there was no correlation with platelet count and FBG or HbA1c. There was also no relationship between platelet count and LDL-C, HDL-C or duration of diabetes. There was however a significant positive correlation between platelet count and BMI (p=0.019; Table 4.3). There was also a trend towards a positive correlation with triglycerides, though not significant (p=0.096). To assess whether hyperglycaemia was associated with platelet size in the diabetic cohort, FBG and HbA1c were correlated with MPV, PDW and P-LCR. There was a significant positive correlation between HbA1c and all measures of platelet size tested (MPV p=0.040; PDW p=0.033; and P-LCR p=0.039). In contrast, FBG did not correlate with any of the measures of platelet size (Table 4.3).

Correlations with other biochemical and demographic characteristics associated with diabetes revealed no relationship between platelet size and LDL-C, HDL-C, triglycerides or BMI. However, there was a strong negative relationship for the duration of diabetes and MPV (p=0.002, PDW p=0.004 and P-LCR, p=0.003, Table 4.3).

Table 4.3: Correlations for platelet indices and biochemical parameters in type 2 diabetic patients

	Platelet count		*MPV*		*PDW*		*P-LCR*	
Variable	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value
Age	-0.096	0.559	-0.148	0.363	-0.157	0.332	-0.152	0.350
HbA1c	-0.111	0.495	**0.106**	**0.040***	**0.338**	**0.033***	**0.328**	**0.039***
FBG	-0.252	0.117	0.166	0.298	0.178	0.272	0.177	0.276
LDL-C	0.169	0.298	-0.033	0.835	-0.077	0.638	-0.039	0.809
HDL-C	-0.118	0.468	0.126	0.434	0.019	0.909	0.101	0.536
Triglycerides	0.267	0.096	-0.083	0.604	-0.171	0.291	-0.048	0.770
BMI	**0.370**	**0.019***	-0.184	0.257	-0.301	0.059	-0.167	0.302
Duration of diabetes	0.135	0.412	**-0.412**	**0.009****	**-0.454**	**0.004****	**-0.418**	**0.008****

Pearson correlation coefficient used to calculate the correlations for HbA1c, LDL-C, triglycerides and BMI. All other correlations calculated using the Spearman test. * represents *p*=<0.05. ** represents *p*=<0.005. *** represents *p*=<0.0005. All tests n=40, except duration of diabetes (n=39).

It has been shown that type 2 diabetic patients with an HbA1c above 64 mmol/mol are more at risk of macrovascular complications (van Wijingaarden et al., 2017). Consequently, an independent t-test was performed to determine if patients with HbA1c $\geq$64 mmol/mol had an elevated MPV. The mean $\pm$ SD MPV was 10.47 $\pm$ 0.23 fL for $\leq$63 mmol/mol and was significantly higher at 11.32 $\pm$ 0.20 fL for $\geq$64 mmol/mol (p=0.009; Figure 4.1A).

To determine whether there was a significant difference in MPV between higher and lower FBG levels, the patient samples were grouped into those ≤8.9 mmol/L and those ≥9.0 mmol/L. These values were based on evidence from a large meta-analysis, which identified 9.0 mmol/L FBG as a level that increased the relative risk of cardiovascular events from 1 to 2.2 (Coutinho et al., 1999). Results showed no significant difference in MPV between the lower and higher FBG groups (10.83 ± 0.22 fL and 10.98 ± 0.26 fL, respectively, p=0.811; Mann-Whitney, Figure 4.1B).

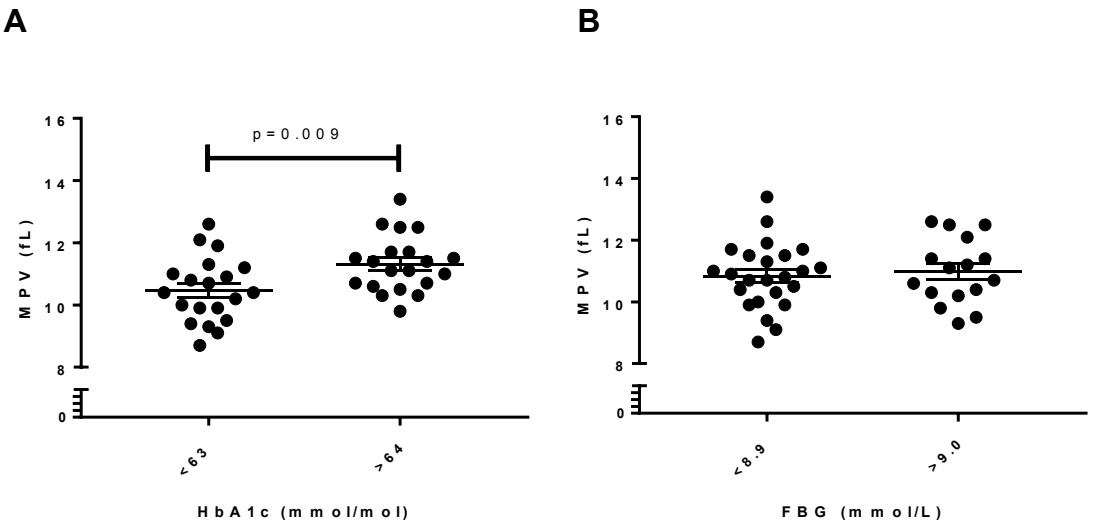

Figure 4.1: Comparison of MPV in type 2 diabetic patients with high and low glucose levels

Figure A: An independent t-test identified a significant increase in MPV in patients with HbA1c ≥64 mmol/mol (p=0.009, n=20, 20). **Figure B:** A Mann-Whitney test showed no significant difference in MPV in patients with FBG ≥9.0 mol/L (p=0.811) (n=24, 16). Bars represented as mean ± SEM.

4.2.3 Thrombopoiesis

Larger, younger platelets are metabolically and enzymatically more active, and have greater prothrombotic potential (Chu et al., 2009). Furthermore, they are often immature platelets that contain residual mRNA and a greater number of dense granules (Mijovic et al., 2015). A larger number of immature platelets in the platelet population reflects increased thrombopoiesis (Hannawi et al., 2018). To assess whether increased thrombopoiesis and a larger number of immature platelets contributes to the increased platelet size observed with hyperglycaemia (Table 4.3), the percentage of reticulated platelets (known as 'IPF') was correlated with HbA1c and FBG in a subset of twenty of the type 2 diabetic patients recruited to the study (see Table 4.1 for baseline characteristics of the cohort).

The mean IPF for the diabetic cohort was 12.14 $\pm$ 8.56 % (n=20) and the mean platelet count was 245.0 $\pm$ 51.7 $x10^9$/L. There was no correlation between the platelet count and reticulated platelets (r=0.057, n=20, p=0.811).

Correlations with other diabetic-related parameters however showed that reticulated platelets positively correlated with LDL-C in type 2 diabetic patients (p=0.044) (Table 4.4). No correlation was observed with any other clinical parameter.

Table 4.4: Correlations for reticulated platelets and clinical parameters in type 2 diabetic patients

	Reticulated platelets	
Variable	*R*	*P* value
Age	0.345	0.136
HbA1c	-0.144	0.545
FBG	-0.762	0.762
LDL-C	**0.454**	**0.044***
HDL-C	0.324	0.164
Triglycerides	0.124	0.603
BMI	-0.095	0.691
Duration of diabetes	0.207	0.381

All correlations calculated using the Spearman test. * represents p=<0.05, n=20.

4.2.4 Platelet Activation Markers

Data related to platelet activation markers in diabetic patients without prior CV events is limited and conflicting (Shlomai et al., 2015). To investigate the activation state of circulating platelets in type 2 diabetic patients, α-granule secretion (CD62P) and $\alpha_{IIb}\beta_3$ activation (PAC1 binding) was measured in resting and activated platelets. Due to technical failures with the flow cytometer, measurements were performed on thirty of the forty diabetic patients recruited (see Table 4.1 for baseline characteristics for the forty patients). In resting platelets, the mean fluorescent intensity (MFI) representing CD62P membrane expression was 25.03 $\pm$ 19.69 in resting platelets, with a significant increase to 1422.53 $\pm$ 497.86 following ADP stimulation (5.0 µmol/L) (Mann Whitney test, p=<0.0001) (Figure 4.2A). PAC-1 levels significantly increased from a resting level of 11.66 $\pm$ 5.00 to 321.0 $\pm$ 98.30 following ADP stimulation (5.0 µmol/L) (Mann Whitney test, p=<0.0001) (Figure 4.2B).

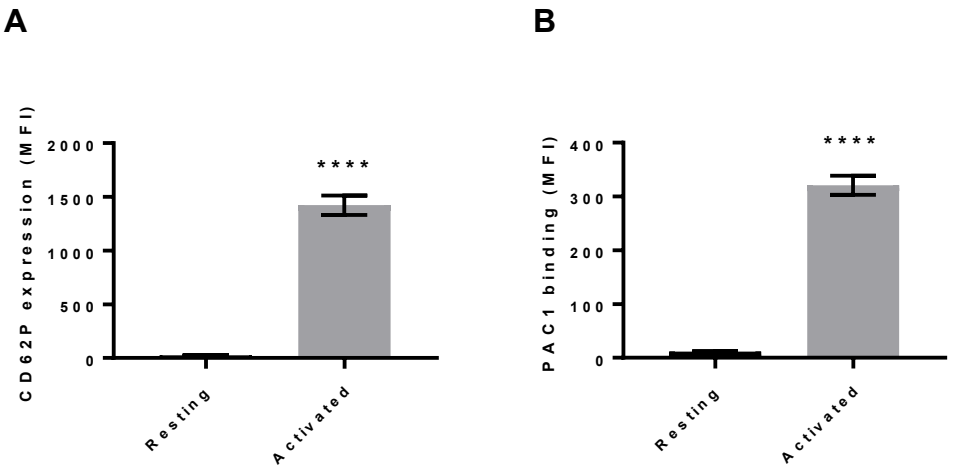

Figure 4.2: Activation markers in resting and ADP-activated platelets in type 2 diabetic patients

Figure A: Mean CD62P surface expression (MFI) in resting and ADP-activated platelets (ADP 5.0 µmol/L) type 2 diabetic patients. **Figure B:** Mean PAC-1 binding (MFI) in resting and ADP-activated platelets (ADP 5.0 µmol/L) type 2 diabetic patients. Values represented as mean ± SEM. **** represents p=<0.0001 (n=30).

CD62P expression in resting platelets ranged from 6 to 99 MFI and in activated platelets ranged from 289 to 2536. Correlations with the clinical parameters of the patients showed no correlation with any of the parameters and the extent of CD62P exposure in resting or ADP-activated platelets (Table 4.5). Although there was a close to significant relationship between the duration of diabetes and CD62P expression in activated platelets (*p*=0.052). PAC-1 binding in resting and ADP-activated platelets ranged from 1 to 22; and 289 to 580, respectively, within the diabetic cohort. Similar to CD62P, there was no correlation with any of the clinical parameters assessed and extent of $\alpha_{IIb}\beta_3$ activation (Table 4.5).

Table 4.5: Correlations for platelet activation markers and clinical parameters in type 2 diabetic patients

	CD62P: Resting platelets		*PAC-1 Resting platelets*		*CD62P: Activated platelets*		*PAC-1: Activated platelets*	
Variable	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value
Age	0.072	0.706	-0.089	0.640	0.326	0.079	0.145	0.446
HbA1c	0.193	0.306	-0.130	0.493	0.059	0.758	-0.024	0.898
FBG	0.289	0.122	-0.088	0.644	-0.083	0.661	-0.077	0.688
LDL-C	0.065	0.735	-0.287	0.125	-0.019	0.919	-0.177	0.349
HDL-C	-0.068	0.720	-0.333	0.072	0.102	0.591	-0.003	0.987
Triglycerides	-0.010	0.959	-0.081	0.671	-0.188	0.320	-0.221	0.241
BMI	0.092	0.628	-0.143	0.452	0.154	0.415	-0.243	0.196
Duration of diabetes	0.260	0.165	-0.194	0.305	0.359	0.052	0.011	0.955

All correlations calculated using Pearson, except those with the variables HbA1c, LDL-C and HDL-C, and correlations for CD62P expression in resting platelets, which used the Spearman test (n=30).

4.2.5 Soluble P-selectin

Circulating sP-selectin (originating from endothelial cells and platelets) has been implicated in the development of atherosclerotic lesions (Burger and Wagner, 2003), and elevated levels of sP-selectin have been found in diabetic patients (Kopp et al., 1998). sP-selectin levels have also been shown to be a good marker of *in vivo* platelet activation status (Ferroni et al., 2009). To determine whether there was a relationship between *in vivo* platelet activation state and any of the clinical characteristics associated with the diabetic patients, sP-selectin was measured and correlated with the clinical parameters of the group.

The mean sP-selectin for the 40 patients was 5275.99 $\pm$ 2184.91 pg/ml, with a range of 2481.88 to 14514.01 pg/ml. Table 4.6 shows the correlations for sP-selectin and clinical parameters. There was a significant positive correlation for sP-selectin and duration of diabetes (p=0.034). All other variables did not significantly correlate with sP-selectin.

Table 4.6: Correlations for sP-selectin and clinical parameters in type 2 diabetic patients

	Soluble P-selectin	
Variable	*R*	*P* value
Age	0.176	0.277
HbA1c	-0.101	0.536
FBG	-0.031	0.850
LDL-C	-0.105	0.519
HDL-C	-0.087	0.593
Triglycerides	0.141	0.385
BMI	0.228	0.157
Duration of diabetes[$]	**0.340**	**0.034***

All correlations calculated using the Pearson test, except HDL-C (Spearman). * represents *p*=<0.05, n=40 (except variable denoted [$] with n=39).

4.2.6 Platelet Aggregation

Light transmission aggregometry (LTA) is the gold standard method for assessing platelet function. To investigate whether platelet aggregation is associated with clinical characteristics associated with diabetes (e.g. hyperglycaemia, dyslipidaemia and BMI), PRP from diabetic subjects was stimulated with submaximal concentrations of physiological platelet agonists collagen or ADP.

Plasma was available for thirty-five of the forty diabetic patients recruited to perform LTA. A summary of the baseline characteristics for the type 2 diabetic patients are given in Table 4.1.

Platelet aggregation was measured in response to submaximal concentrations of ADP (5.0 µmol/L) and collagen (1.0 µg/ml), elucidated from a dose response (Appendix 9.1). Maximal aggregation for ADP was 76.23 $\pm$ 14.07 % following 5 minutes stimulation and 81.80 $\pm$ 6.69 % for collagen. To investigate whether the level of agonist induced platelet reactivity in the diabetic cohort was related specifically to anyone of the clinical parameters measure a series of correlations were performed (Table 4.7).

Data showed no correlation between the extent of ADP or collagen-induced aggregation and HbA1c or FBG further supporting the notion, that hyperglycaemia does not enhance platelet reactivity. The data did however show a strong positive correlation with LDL-C levels and the maximal aggregation achieved with ADP (Spearman, r=0.501, p=0.002) and collagen (Spearman, r=0.535; p=0.001) (Figure 4.3A and B). In addition, there was a significant

negative correlation for BMI and ADP-stimulated aggregation. (Spearman, r=-0.426, n=35, p=0.011).

Table 4.7: Correlations between platelet aggregation and clinical parameters in type 2 diabetic patients

	ADP-activated aggregation (maximum %)		*Collagen-activated aggregation (maximum %)*	
Variable	*R*	*P* value	*R*	*P* value
Age	-0.027	0.876	0.012	0.946
HbA1c	-0.162	0.354	-0.078	0.055
FBG	-0.063	0.718	0.122	0.485
LDL-C	**0.501**	**0.002****	**0.535**	**0.001****
HDL-C	0.266	0.122	0.220	0.205
Triglycerides	-0.117	0.504	0.114	0.514
BMI	**-0.470**	**0.004****	-0.273	0.113
Duration of diabetes	-0.264	0.125	-0.294	0.087

Pearson correlation coefficient used to calculate the correlations for age, triglycerides, BMI and duration of diabetes. All other correlations, including those correlated against collagen, were calculated using the Spearman test. ** represents *p*=<0.005 (n=35).

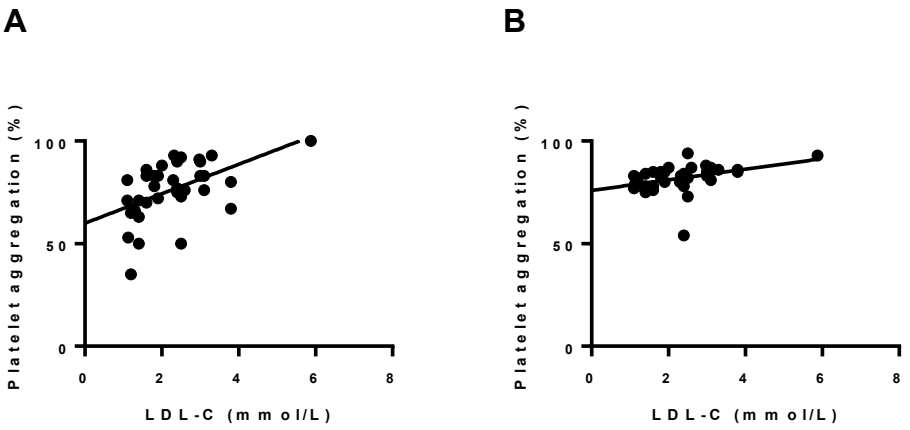

Figure 4.3: The relationship between LDL-C and maximal platelet aggregation in type 2 diabetic patients
Figure A: represents the correlation between LDL-C and ADP-stimulated aggregation in type 2 diabetic patients (Spearman, p=0.002, n=35). **Figure B:** represents the correlation between LDL-C and collagen-stimulated aggregation (Spearman, p=0.001, n=35).

Although NICE guidelines recommend using non-HDL-C to assess cardiovascular risk (NICE, 2018), guidance from the Joint British Societies (2005), recommended that people at high risk of cardiovascular disease, including diabetic patients, should aim for a target LDL-C of < 2.0 mmol/L and the NHS states that diabetic patients should have LDL-C <2.0 mmol/L (NHS, 2018). To assess whether platelet reactivity is significantly altered between LDL-C levels below and above 2.0 mmol/L, aggregation responses to ADP and collagen were grouped based of LDL-C plasma concentration and a Mann-Whitney U test performed to statistically compare responses (Figure 4.4A and Figure 4.4B). Patients with LDL-C above 2.0 mmol/L demonstrated significantly increased platelet aggregation in response to both ADP- and collagen (p=0.005 and p=0.016, respectively).

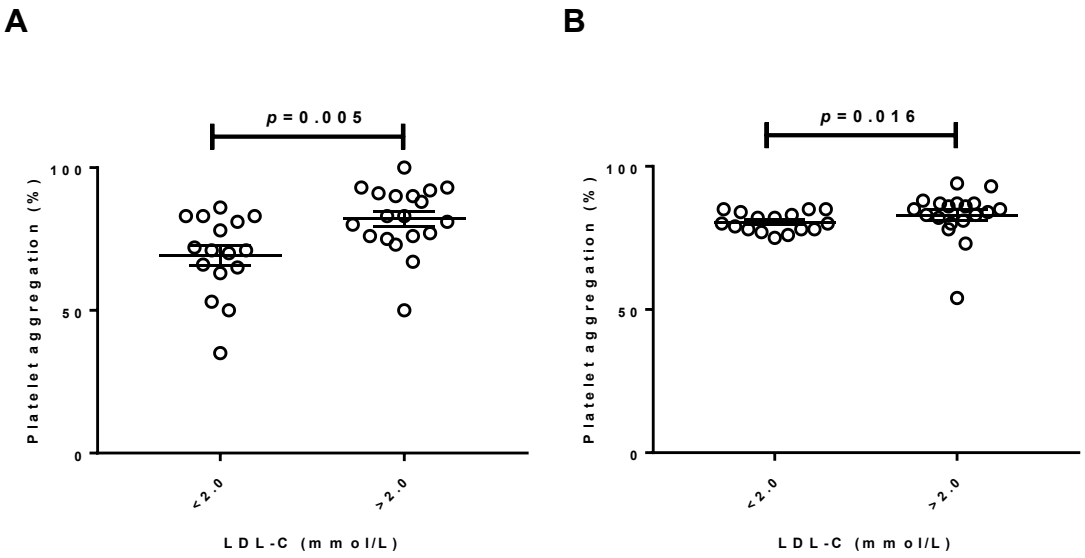

Figure 4.4: Maximal platelet aggregation in type 2 diabetic patients with LDL-C levels above and below 2.0 mmol/L

Figure A: represents ADP-stimulated platelet aggregation in type 2 diabetic patients with LDL-C <2.0 mmol/L and >2.0 mmol/L. The mean ± SEM for platelet aggregation were 69.38% ± 3.51% and 82.0% ± 2.65%. **Figure B:** represents collagen-stimulated platelet aggregation in type 2 diabetic patients with LDL-C <2.0 mmol/L and >2.0 mmol/L. The mean ± SEM for platelet aggregation were 80.44% ± 0.84% and 82.95% ± 1.95%. P values calculated using the Mann-Whitney U test for non-parametric data. Bars represented as mean ± SEM (n=16, n=19).

4.2.7 Oxidised LDL

The correlation data from section 4.2.6 identifies LDL-C as the most important biochemical risk factor for platelet hyperreactivity in type 2 diabetic patients. Literature suggests that circulating ox-LDL is increased in type 2 diabetic patients (Nakhjavani et al., 2010) and that platelet hyperreactivity is associated with ox-LDL (Podrez et al., 2007). However, research also indicates that platelet hyperreactivity is induced by ox-LDL via an indirect effect caused by modulation of cGMP signalling (Magwenzi et al., 2015). In this study, we investigated whether there is a relationship between ox-LDL levels and the clinical parameters associated with the diabetic cohort and also whether ox-LDLC levels are related to platelet responsiveness to agonists.

An ox-LDL ELISA was performed on the plasma samples for the forty diabetic patients recruited in this study. The ox-LDL levels were detected within the standard curve for thirty-one of the forty plasma samples. The mean $\pm$ SD ox-LDL for these samples was 1161.27 pg/ml $\pm$ 188.95 pg/ml.

Correlations were carried out with ox-LDL (pg/ml) concentrations against clinical characteristics and platelet reactivity. Research suggests that the ox-LDL/LDL-C and ox-LDL/HDL-C ratios can be used as predictors of CAD (Huang et al., 2008), therefore correlations were also performed with the ox-LDL/LDL-C and ox-LDL/HDL-C ratios.

A positive correlation with BMI and both ox-LDL/LDL-C ratio ($p=0.006$) and ox-LDL/HDL-C ratio ($p=0.020$) (Table 4.8 and Figure 4.5A).

The ox-LDL levels did not significantly correlate with ADP-stimulated aggregation (p=0.279) or collagen-stimulated aggregation (p=0.567) (Table 4.9). Paradoxically, there was a strong inverse correlation between ox-LDL/LDL-C and ADP and collagen-stimulated aggregation (p=<0.00003 and p=0.0003, respectively) (Table 4.9 and Figure 4.5C and Figure 4.5D). There was also a negative association between ADP-stimulated platelet aggregation and ox-LDL-C/HDL-C ratio (p=0.010).

Additionally, there was a strong negative association with CD62P surface expression and ox-LDL in resting platelets (p=0.004) (Table 4.9 and Figure 4.5B).

Table 4.8: Correlations between clinical parameters and ox-LDL, ox-LDL/LDL-C and ox-LDL/HDL-C in type 2 diabetic patients

	Ox-LDL		*Ox-LDL/LDL-C*		*Ox-LDL/HDL-C*		*N*
Variable	R	P value	R	P value	R	P value	
Age	-0.130	0.486	-0.148	0.426	-0.230	0.214	31
HbA1c	0.012	0.949	0.058	0.755	0.137	0.462	31
FBG	-0.189	0.307	-0.147	0.431	-0.006	0.974	31
BMI	-0.065	0.728	**0.479**	**0.006****	**0.417**	**0.020***	31
Duration of diabetes	-0.186	0.318	0.209	0.200	0.299	0.215	31

All correlations calculated using the Pearson coefficient, except correlations with FBG which was calculated using the Spearman test. * represents $p \leq 0.05$, ** represents $p \leq 0.01$.

Table 4.9: Correlations between platelet reactivity and ox-LDL in type 2 diabetic patients

	Ox-LDL		*Ox-LDL/LDL-C*		*Ox-LDL/HDL-C*		*N*
Platelet count	-0.110	0.557	-0.188	0.311	0.210	0.257	31
MPV	-0.255	0.167	-0.043	0.820	-0.196	0.291	31
CD62P in resting platelets	**-0.632**	**0.004****	-0.303	0.208	-0.129	0.600	19[$]
PAC-1 in resting platelets	0.028	0.909	0.218	0.370	0.151	0.538	19[$]
CD62P in activated platelets	-0.008	0.974	0.013	0.957	0.207	0.395	19[$]
PAC-1 in resting platelets	0.210	0.389	-0.168	0.492	0.183	0.452	19[$]
ADP-stimulated aggregation	-0.201	0.279	**-0.679**	**0.00003****	**-0.455**	**0.010***	31
Collagen-stimulated aggregation	-0.107	0.567	**-0.607**	**0.0003****	-0.147	0.430	31

All correlations calculated using the Pearson coefficient, except correlations with FBG, CD62P expression in resting platelets and collagen-stimulated aggregation which were calculated using the Spearman test. * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and **** represents $p \leq 0.0001$. [$] 19 samples were correlated with CD62P and PAC-1 binding due to technical problems with the flow cytometry.

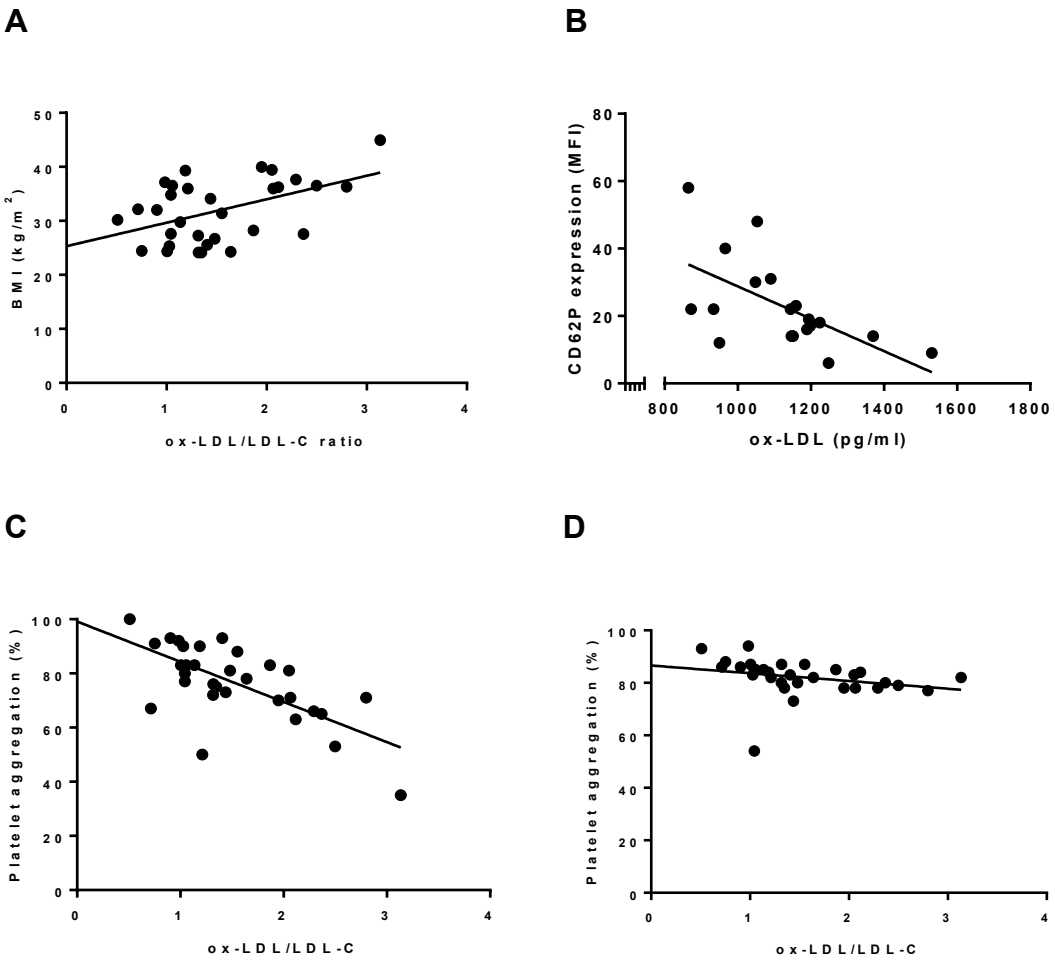

Figure 4.5: Correlations for ox-LDL in type 2 diabetic patients
Figure A: represents a Pearson correlation for ox-LDL/LDL-C and BMI (r=0.479, n=31, p=0.006). **Figure B:** represents a Spearman correlation for ox-LDL and CD62P membrane expression in resting platelets (r=-0.632, n=19, p=0.004). **Figure C:** represents a Pearson correlation for ox-LDL/LDL-C and ADP-stimulated platelet aggregation (r=-0.0679, n=31, p=0.00003). **Figure D:** represents a Spearman correlation for ox-LDL/LDL-C and collagen-stimulated platelet aggregation (r=-0.0607, n=31, p=0.0003).

4.3 Discussion

This study has shown that elevated platelet count is associated with BMI in diabetic patients (p=0.019), with a trend towards an elevated platelet count with increased triglycerides (p=0.096). This supports research by Akinsegun et al. (2014) who also identified a positive relationship between BMI and platelet count in a type 2 diabetic cohort. Samocha-Bonet et al. (2008) identified elevated platelet counts in obese subjects, particularly in subjects with elevated C-reactive protein levels (a marker of inflammation). They suggested that elevated platelet count is associated with chronic inflammation due to higher body fat mass. A large amount of the literature suggests that inflammatory mediators are associated with obesity (Wellen and Hotamisligil, 2005), and platelets are now being considered as key players in inflammation (Stokes and Granger, 2012).

MPV is positively correlated with HbA1c levels in type 2 diabetic patients, indicating that hyperglycaemia is associated with increased platelet size. This supports the *in vitro* data in section 3.1 and the literature which shows an association between HbA1c and MPV *ex vivo* (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder et al, 2014; Ulutas et al., 2014). Furthermore, patients with HbA1c $\geq$64 mmol/L have significantly elevated MPV. Conversely, FBG does not correlate with type 2 diabetes, and this supports the work by Demirtunc et al. (2009) and Hekimsoy et al. (2004) who reported a correlation between MPV and HbA1c, and no correlation between MPV and FBG. The increased platelet size does not appear to be related to increased platelet reactivity. Given that a) we observed an effect *in vitro*, independent of any contribution from thrombopoiesis and b) there was no relationship between hyperglycaemia and IPF in diabetic

samples, the increase in platelet volume evoked by hyperglycaemia is likely through osmotic/diffusion effects. This phenomenon has also been observed in erythrocytes by uptake of glucose via the GLUT transporters (Lösche et al., 1989).

Unexpectedly, we have demonstrated that MPV is inversely related to the duration of diabetes. The majority of patients in this study are taking statins and anti-diabetic medication such as metformin, and research suggests that statins decrease MPV (Sivri et al., 2013; Xian-Yu et al., 2015; Akyüz et al., 2016) as well as metformin (DolasıK et al., 2013). It could be postulated that the longer the patients have diabetes, the longer they take this medication and the more the MPV will decrease. Another reason for the inverse correlation could be due to platelet consumption at the sites of high-level inflammation that correspond to diabetic complications, such as renal failure (Bilen et al., 2015). Another explanation is that patients with late stage diabetes have more vascular disease and there is an increase *in vivo* platelet activation demonstrated by elevated sP-selectin levels (Kopp et al., 1998). Larger more active platelets are therefore immobilised in vessels leaving the smaller, less active platelets in the circulation. This explanation is supported by the sP-selectin correlation data (Section 4.2.5) that showed a significant relationship between sP-selectin and duration of diabetes. This corroborates findings from Aref et al. (2005) that showed insignificantly elevated sP-selectin in early-stage diabetic patients (without CAD) compared to controls, and significantly elevated sP-selectin in late stage diabetes with CAD.

A positive correlation was identified with LDL-C and reticulated platelets. This indicates that high levels of LDL-C increase the thrombopoiesis, particularly since there was no overall increase in platelet number. Research has shown that younger platelets are metabolically and enzymatically more active, and have greater prothrombotic potential (Mijovic et al., 2015). Furthermore, diabetic patients do not respond well to antiplatelet therapy such as aspirin, and it is thought that this is due to the high levels of immature platelets because the newly released platelets have not been inhibited by the aspirin (Guthikonda et al., 2007). Consequently, the results from this study suggest that elevated LDL-C levels can increase the levels of immature platelets, and this in turn reduces the patient's response to the aspirin.

The key finding from this study is that there is a strong positive correlation between LDL-C and both ADP and collagen-stimulated platelet aggregation. This supports the results for the correlations with thrombopoiesis and LDL-C. Potentially, the high level of younger, more metabolically active platelets is increasing their ability to aggregate, and this is dependent on the levels of LDL-C in the circulation.

There were no significant correlations for the platelet activation markers, CD62P and PAC-1. Considering PAC-1 binding measures $\alpha_{IIb}\beta_3$ activation, the final step towards platelet aggregation, one would assume that PAC-1 binding would correlate with LDL-C.

When we investigated the relationship between ox-LDL and platelet reactivity, we identified a negative relationship between ox-LDL/LDL-C and platelet aggregation. This would indicate that it is native LDL that is potentiating the platelet aggregation in type 2 diabetic patients, not ox-LDL. Additionally, BMI negatively correlates with platelet aggregation but positively correlates with ox-LDL/LDL-C, demonstrating that elevated BMI decreases platelet aggregation because the n-LDL is converted to ox-LDL.

Most of the literature has pointed to ox-LDL as a reason for platelet hyperreactivity, however, we have shown a negative correlation with ox-LDL/LDL-C and a positive correlation with LDL-C. It is possible that the native LDL is potentiating platelet aggregation, however, further research is required to investigate this. Other literature has shown that cholesterol accumulation disturbs the composition of the membrane in various hematopoietic cells and enhances receptor signalling (Zhu et al., 2010; Yvan-Charvet et al, 2010). Increased expression of $P2Y_{12}$ has been reported in diabetic patients (Hu et al., 2017). Thus, plasma membrane cholesterol accumulation in platelets could potentially alter the membrane structure and affect signalling via surface receptors, such as $P2Y_{12}$. This, in turn, would reduce the ability for antiplatelet therapies (such as clopidogrel) to work effectively.

To the best of our knowledge, this is the first study to identify LDL-C as a risk factor for platelet hyperreactivity in type 2 diabetic patients, and that LDL-C may act as a biomarker to identify type 2 diabetic patients who would benefit from antiplatelet prophylaxis. Additionally, enhanced platelet turnover mediated by

elevated LDL-C may be responsible for reduced antiplatelet efficacy in type 2 diabetic patients.

5 Identification of Inflammatory Mediators Associated with Platelet Reactivity in Type 2 Diabetic Patients

5.1 Introduction

The majority of type 2 diabetic patients become insulin resistant because of obesity and physical inactivity, and this is associated with chronic inflammation (Wellen and Hotamisligil, 2005). The proposed mechanism that causes low-level inflammatory is increased adipose tissue and hyperglycaemia, which leads to oxidative stress and the accumulation of macrophages in the endothelium (Wellen and Hotamisligil, 2005). Macrophages release proinflammatory cytokines that alter insulin signalling pathways, enhancing insulin resistance and impaired glucose homeostasis (Badawi et al., 2010). Consequently, type 2 diabetic patients have differing cytokine profiles to healthy people (Al-Shukaili et al., 2013).

Accumulating studies are showing that platelets have (thrombo)-inflammatory responses (Stokes and Granger, 2012). As platelets move through the vasculature of inflamed tissue, inflammatory mediators can activate platelets such as cytokines and chemokines (Gleissner et al., 2008). Also, platelets can themselves initiate inflammatory responses through their receptors (Vieira-de-Abreu et al., 2012).

To investigate the relationship between platelet reactivity and inflammation in type 2 diabetes, we used a multiplex immunoassay to simultaneously measure inflammatory mediators including cytokines, chemokines and growth factors. The inflammatory profile of the plasma was measured using a panel of twenty inflammatory molecules, of these ten had plasma concentration in the detectable

range in fifteen samples or more (Table 5.1). Inflammatory markers detected in less than fifteen samples were not included in the correlations.

Table 5.1: Inflammatory markers used in the immunoassay

Target protein	***Anti- or pro-atherogenic***	***Roles in atherosclerosis and diabetes***	***Roles in platelet function and production***
E-selectin	Pro	Expressed on endothelial cells. AGEs bind to RAGE on endothelial cells and induce expression of E-selectin (Basta et al., 2002).	Knockout of P-selectin and E-selectin decreases atherosclerosis in murine models (Collins et al., 2000).
IL-1β	Pro	Activation of macrophages and endothelial cells (Fatkhullina et al., 2016). Increased expression associated with hyperglycaemia (Butkowski and Jelinek, 2016).	Synthesised in platelets (Vieira de Abreu et al., 2012). Stimulates thrombopoiesis (Jiang et al., 1994).
IL-12p70	Anti	Active heterodimer of the inflammatory cytokine, IL-12 (Gee et al., 2009). Involved in anti-angiogenesis.	IL-12 caused platelets to be hyperreactive and spread (Page et al., 2018).
IL-13	Anti	Inhibits foam cell formation by Inhibiting MCP-1α transcription in macrophages (Berkman et al., 1996).	Expressed by a human megakaryocyte-like cell line (Soslau et al., 1997).

Table cont.			
IL-6	Pro	Foam cell formation (Fatkhullina et al., 2016). Increased expression associated with hyperglycaemia (Butkowski and Jelinek, 2017).	Stimulates thrombopoiesis (Jiang et al., 1994).
IP-10	??	Inhibition of angiogenesis by IL-12 is mediated by IP-10 (Sgadari et al., 1996). Chemoattractant for monocytes and lymphocytes (Taub et al., 1993).	Mediates IL-12 activation (10 (Sgadari et al., 1996). IL-12 caused platelets to be hyperreactive and spread (Page et al., 2018).
MCP-1 (CCL2)	Pro	Chemoattractant for monocytes (Deshmane et al., 2009). Contributes to macrophage recruitment and insulin resistance (Kamei el., 2006).	Secreted by endothelial cells in the presence of activated platelets (Cha et al., 2000).
MIP1α (CCL3)	Pro	Foam cell formation (Berkman et al., 1996).	Stored in platelets (Klinger et al., 1995). Secreted by endothelial cells in the presence of activated platelets (Cha et al., 2000).
sICAM-1	Pro	Promotes adhesion of leukocytes to the endothelium. AGEs bind to RAGE on endothelial cells and induce expression of ICAM-1 (Basta et al., 2002).	Activated platelets bind to endothelium via ICAM-1 (Bombelli et al., 1998)

Table cont.			
TNF-α	Pro	Deficiency in TNF-α reduces foam cell formation and the expression of several proinflammatory markers.	TNF-α triggers TXA_2 activation (Pignatelli et al., 2005).

IL: Interleukin; IP: Interferon gamma-induced protein, MCP: Monocyte chemoattractant protein, sICAM: soluble intercellular adhesion molecule, TNF: Tumor necrosis factor.

In this chapter, the aim was to determine whether the inflammatory status of the diabetic cohort was related to any of the other clinical characteristic associated with type 2 diabetes (e.g. glycaemic control, lipoprotein levels or BMI) or whether it was independent of these parameters. More importantly, correlations were performed to evaluate whether inflammatory status was related to platelet reactivity or the activation state of platelets *ex vivo*, and more specifically which cytokines or inflammatory markers.

5.2. Results

5.2.1 Inflammatory Mediators and Clinical Parameters

Diabetes and obesity are associated with chronic inflammation induced by oxidative stress (De Bandiera et al., 2013). This may be caused by AGE-RAGE signalling pathways activating NFκβ which, in turn, triggers cytokine transcription and leukocyte recruitment to the vascular wall (De Bandiera et al., 2013, Nonaka et al., 2018). Moreover, Butkowski and Jelinek (2017) identified several inflammatory markers associated with hyperglycaemia including IL-1β, IL-6, IL-10 and MCP-1. Conversely, a study reported that elevated fatty acids induce an increase in inflammatory adhesion molecules (e.g. ICAM-1), not hyperglycaemia, in an endothelial cell line mimicking diabetic retinopathy (Chen et al., 2003).

To investigate the inflammatory status of the diabetic cohort and determine whether it was related to any of the other diabetes-related characteristics of the cohort, an inflammatory cytokine multiplex assay was performed on the patient plasma samples and correlated with the clinical parameters. The mean plasma concentrations of each of the ten markers measured are shown in Table 5.2. Correlation data demonstrated that HbA1c negatively correlated with IL-1β and positively correlated with IP-10 ($p=0.013$ and $p=0.045$, respectively) (Table 5.3). Age negatively correlated with E selectin and positively correlated with IL-1β ($p=0.030$ and $p=0.013$, respectively). HDL-C negatively correlated with E-selectin and siCAM1 ($p=0.021$ and $p=0.013$, respectively). There was a negative association with IL-13 and triglycerides ($p=0.045$).

Table 5.2: Mean values for inflammatory mediators in type 2 diabetic patients

Inflammatory marker	*Mean ± SD (pg/ml)*	*N*
E-selectin	46115.45 ± 6695.58	40
IL-1β	160.77 ± 147.25	26‡
IL-12p70	809.87 ± 574.35	36‡
IL-13	177.62 ± 271.05	32‡
IL-6	2933.13 ± 2590.38	23‡
IP-10	288.85 ± 294.66	40
MCP-1	414.85 ± 200.74	40
MCP-1α	302.27 ± 312.34	26‡
sICAM-1	30701.51 ± 15124.68	40
TNFα	1515.16 ± 858.00	40

‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection.

al characteristics correlated with inflammatory mediators

	Age		*HbA_{1c}*		*FBG*		*BMI*		*DoD*[$]		*LDL-C*		*HDL-C*		*Trig.*	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
E-selectin	**-0.343**	**0 .030***	0.167	0.303	-0.142	0.384	0.281	0.079	0.119	0.466	-0.034	0.836	**-0.364**	**0.021***	0.259	0.107
IL-1β[‡]	**0.478**	**0.013***	**-0.482**	**0.013***	-0.141	0.433	-0.076	0.711	-0.007	0.975	-0.099	0.631	0.138	0.502	-0.258	0.204
IL-12p70[‡]	0.313	0.063	-0.122	0.480	-0.116	0.520	-0.135	0.433	0.214	0.210	-0.182	0.289	-0.004	0.980	-0.110	0.524
IL-13[‡]	0.072	0.694	-0.041	0.822	0.053	0.765	-0.152	0.408	-0.037	0.841	0.070	0.704	-0.069	0.707	**-0.356**	**0.045***
IL-6[‡]	0.263	0.885	-0.219	0.315	0.088	0.699	0.244	0.263	0.162	0.460	-0.065	0.769	-0.184	0.402	-0.256	0.239
IP-10	-0.024	0.882	**0.318**	**0.045***	0.211	0.192	0.060	0.714	0.138	0.395	-0.233	0.147	0.051	0.753	0.147	0.661
MCP-1	0.231	0.152	-0.281	0.079	-0.024	0.885	0.104	0.524	0.275	0.086	-0.098	0.546	0.055	0.735	-0.222	0.168
MCP-1α[‡]	-0.261	0.198	0.386	0.051	0.378	0.057	0.281	0.078	0.158	0.440	-0.042	0.839	-0.205	0.315	0.029	0.888
sICAM-1	-0.069	0.674	-0.049	0.765	-0.149	0.360	0.120	0.460	0.332	0.036	-0.143	0.378	**-0.389**	**0.013***	0.030	0.856
TNFα	0.291	0.068	-0.052	0.751	0.060	0.714	-0.140	0.390	0.278	0.083	-0.003	0.985	0.132	0.416	-0.173	0.285

[$] denotes 1 missing value, no record of duration of diabetes on patient record. E. gamma-induced protein, MCP: Monocyte chemoattractant protein, sICAM: soluble necrosis factor. N=40 (some cytokine data was outside the limit of detection).

5.2.2 Inflammatory Mediators and Platelet Indices

Diabetic patients have elevated platelet count (Sterner et al., 1998) and increased immature platelets (Mijovic et al., 2015). It has been reported that the larger, more reactive platelets migrate to inflammatory sites where they are consumed, and this enhances thrombopoiesis to increase the quantity of circulating platelets, resulting in a shift in platelet size (such as MPV) and platelet count (Turner-Stokes et al.,1991; Gasparyan et al., 2010). Moreover, inflammatory mediators influence the release of platelets from megakaryocytes including IL-β and IL-6 (Jiang et al., 1994).

To assess whether inflammatory status in diabetic patients was associated with platelet count or size, the panel of inflammatory markers was correlated with the platelet parameters obtained from a full blood count (n=31).

It was determined that platelet size (MPV and P-LCR) correlated with TNF-α ($p=0.041$ and $p=0.045$, respectively) and MCP-1α correlated with platelet count ($p=0.003$) (Table 5.4).

Table 5.4: Correlations for platelet indices and inflammatory markers

	Platelet count		***MPV***		***PDW***		***P-LCR***		***N***
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	
E-selectin	0.125	0.503	-0.037	0.844	-0.137	0.462	-0.126	0.499	31
IL-1β	0.413	0.088	0.274	0.271	0.173	0.493	0.128	0.614	18‡
IL-12p70	-0.277	0.162	0.346	0.775	0.323	0.100	0.345	0.078	27‡
IL-13	-0.060	0.785	0.132	0.548	0.209	0.338	0.238	0.273	23‡
IL-6	-0185	0.509	-0.427	0.112	-0.342	0.213	-0.299	0.280	15‡
IP-10	-0.237	0.200	0.194	0.295	0.176	0.344	0.160	0.391	31
MCP-1	0.182	0.328	-0.183	0.325	-0.209	0.194	-0.122	0.514	31
MCP-1α	**0.652**	**0.003****	-0.257	0.303	-0.227	0.364	-0.184	0.464	18‡
sICAM-1	-0.165	0.374	0.044	0.812	0.025	0.895	0.040	0.830	31
TNFα	-0.025	0.894	**0.370**	**0.041***	0.219	0.237	**0.363**	**0.045***	31

All p values calculated using the Pearson correlation coefficient except the correlations with IL-1β, IP-10, IL-13 and E selectin. ‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection. * denotes $p \leq 0.05$ and ** denotes $p \leq 0.01$.

5.2.3 Inflammatory Mediators and Platelet Activation

Under inflammatory conditions, circulating platelets adhere to activated endothelial cells and recruit leukocytes (Langer et al., 2017). Intravital microscopy in inflamed murine vessels demonstrated that the migration of neutrophils is mediated by the platelet P-selectin receptor and PSGL-1 (Sreeramkumar et al. 2014). In addition, Burger and Wagner (2003) reported that platelet P-selectin influences the secretion of chemokines from monocytes, to stimulate the proliferation of SMCs and promote atherogenesis. Moreover, Massberg et al. (2002) reported that $\alpha_{IIb}\beta_3$ activation was required for firm adhesion of platelets to endothelium.

The levels of CD62P (P-selectin) and PAC-1 binding ($\alpha_{IIb}\beta_3$ activation) in resting platelets were correlated with the inflammatory molecules measured in the plasma (Table 5.5). Significant correlations were observed between CD62P membrane expression and IL-12p70 (p=0.043), MCP-1α (p=0.033), sICAM-1 (p=0.022) and TNFα (p=0.042), demonstrating a positive relationship between these inflammatory pathways and platelet α-granules secretion. No significant correlations were identified between PAC-1 binding and the inflammatory status of the patients.

Table 5.5: Correlations for activation markers in resting platelets and inflammatory markers

	CD62P expression		***PAC-1 binding***		***N***
	R	*P*	*R*	*P*	
E-selectin	0.166	0.419	-0.014	0.946	26
IL-1β	0.271	0.222	-0.128	0.570	22‡
IL-12p70	**0.399**	**0.043***	-0.022	0.913	26
IL-13	0.222	0.298	-0.199	0.351	24‡
IL-6	0.414	0.070	0.082	0.730	20‡
IP-10	-0.180	0.317	-0.048	0.816	26
MCP-1	0.204	0.255	-0.210	0.240	26
MCP-1α	**0.490**	**0.033***	-0.267	0.268	18‡
sICAM-1	**0.448**	**0.022***	0.098	0.586	26
TNFα	**0.402**	**0.042***	-0.163	0.427	26

All p values calculated using the Spearman test for CD62P expression in resting platelets. All p values for PAC-1 binding correlations were calculated using the Spearman test except E-selectin and siCAM-1 which used the Pearson test for parametric data. ‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection. * denotes $p \leq 0.05$.

The levels of sP-selectin in the patient plasma was also correlated with the panel of inflammatory markers, to further investigate the relationship with circulating activation status and inflammation. The mean ± SD for sP-selectin was 5800.62 ± 2595.40 pg/ml, demonstrating a large variation across the cohort. Significant positive correlations were identified with sP-selectin and IL-1β (p=0.005), IL-12p70 (p=0.033), IP-10 (p=0.003), MCP-1 (p=0.006) (Table 5.6).

Table 5.6: Correlations for sP-selectin and inflammatory markers

	sP-selectin		
	R	*P*	*N*
E-selectin	0.196	0.224	40
IL-1β	**0.535**	**0.005****	26‡
IL-12p70	0.301	0.074	36‡
IL-13	0.303	0.092	32‡
IL-6	0.185	0.398	23‡
IP-10	**0.423**	**0.007****	40
MCP-1	**0.396**	**0.012****	40
MCP-1α	-0.087	0.672	26‡
sICAM-1	0.260	0.105	40
TNFα	**0.420**	**0.007****	40

All p values calculated using the Spearman test for non-parametric data. ‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection. * denotes $p \leq 0.05$ and ** denotes $p \leq 0.01$.

Correlations for both ADP (5.0 μmol/L)-induced CD62P expression and PAC-1 binding and plasma inflammatory molecules demonstrated no relationship between inflammatory status and platelet reactivity in the cohort (Table 5.7).

Table 5.7: Correlations for activation markers in ADP-activated platelets and inflammatory markers

	CD62P expression		***PAC-1 binding***		***N***
	R	*P*	*R*	*P*	
E-selectin	0.046	0.822	-0.068	0.740	26
IL-1β	-0.094	0.677	-0.103	0.648	22‡
IL-12p70	0.181	0.377	-0.132	0.520	26
IL-13	-0.270	0.202	-0.130	0.545	24‡
IL-6	-0.068	0.776	-0.095	0.690	20‡
IP-10	0.025	0.904	0.025	0.904	26
MCP-1	-0.058	0.777	0.089	0.666	26
MCP-1α	-0.041	0.871	0.251	0.315	18‡
sICAM-1	0.139	0.822	0.032	0.876	26
TNFα	-0.160	0.434	-0.144	0.484	26

All p values were calculated using the Spearman test except correlations with E-selectin and siCAM-1 which used the Pearson test for parametric data. ‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection.

5.2.4 Inflammatory Mediators and Platelet Aggregation

The levels of platelet aggregation were correlated with the inflammatory molecules measured in the plasma. The mean ± SD for ADP-activated aggregation was 74.68 ± 13.39 % and for collagen was 80.25 ± 9.14 %. No significant correlations with inflammatory markers were identified (Table 5.8).

Table 5.8: Correlations for platelet aggregation and inflammatory markers

	ADP-activated aggregation		***Collagen-activated aggregation***		***N***
	R	*P*	*R*	*P*	
E-selectin	-0.016	0.935	0.042	0.832	28
IL-1β	-0.220	0.430	0.227	0.417	15‡
IL-12p70	-0.024	0.913	0.302	0.152	24‡
IL-13	0.277	0.237	0.017	0.944	20‡
IL-6	0.053	0.869	-0.095	0.770	12‡
IP-10	-0.185	0.345	-0.114	0.565	28
MCP-1	-0.118	0.548	-0.117	0.552	28
MCP-1α	0.245	0.360	-0.168	0.535	16‡
sICAM-1	-0.269	0.166	-0.037	0.853	28
TNFα	-0.016	0.935	0.113	0.566	28

All p values were calculated using the Spearman test. ‡ denotes correlations with missing data due to the protein concentration being below the lower limit of detection.

1.1 Discussion

Components of the immune system are altered in type 2 diabetes, with elevated levels of circulating inflammatory mediators such as IL-6 and IL-1β (Donath and Shoelson, 2011). Recent research shows that platelets play an important role in (thrombo)-inflammatory processes (Stokes and Granger, 2012). This chapter examined the relationship between platelet reactivity and inflammatory mediators in type 2 diabetic patients.

The data demonstrated that both E-selectin and siCAM-1 were negatively associated with HDL-C. A study showed that subjects with low HDL had significantly higher concentrations of inflammatory markers (including siCAM-1 and E-selectin) (Ahmad et al., 2015). They also found higher levels of biomarkers associated with oxidative stress in the low-HDL group. HDL has been shown to protect against the development of atherosclerosis and have antioxidant properties (Barter, 2005). It is also involved in reverse cholesterol transport which is the process of removing excessive free cholesterol from the vessel wall (Ahmad et al., 2015). Thus, HDL-C could be used as an independent predictor of endothelial activation, however, given that this study found no correlation between HDL-C and platelet reactivity (Chapter 4.2), this mechanism may not involve platelet activation.

IL-13 has anti-angiogenetic effects by inhibiting expression of the pro-atherogenic marker, MCP-1α in macrophages, to reduce foam cell formation (Berkman et al., 1996). This study found that IL-3 negatively correlated with triglycerides. A characteristic finding in atherosclerosis is the accumulation of triglycerides in

macrophages, leading to foam cell formation (Feingold et al., 2012). This might suggest that the anti-angiogenic effects of IL-13 are reduced in the presence of high levels of triglycerides in type 2 diabetic patients, elevating macrophage activation and foam cell formation.

The data demonstrated that both platelet count and CD62P expression in resting platelets positively correlated with the chemokine, MIP-1α. This inflammatory mediator is secreted by endothelial cells in the presence of activated platelets (Vieira de Abreu et al., 2012). It is stored in α-granules and released upon platelet activation (Vieira de Abreu et al., 2012). Cha et al. (2000) demonstrated that ADP-activated platelets induced the secretion of MCP-1α from endothelial cells. TNF-α correlated with both CD62P expression in resting platelets and platelet size, and siCAM-1 correlated with CD62P in resting platelets. TNF-α is an inflammatory cytokine that has been linked with obesity and diabetes (Hotamisligil et al (1993). TNF-α has been shown to stimulate the arachidonic acid pathway in heart failure patients (Pignatelli et al., 2005). Furthermore, TNF-α induces the expression of the adhesion molecule, iCAM-1 on endothelium and activated platelets can bind to the endothelial cells via iCAM-1 (Bombelli et al., 1998). Additionally, two studies reported elevated iCAM-1 expression in endothelial cells in the presence of activated platelets (Gawaz et al., 1998; Cha et al., 2000).

This study also showed that IL-12p70 was associated with CD62P expression in resting platelets. IL-12p70 is the active heterodimer of the inflammatory cytokine, IL-12 (Gee et al., 2009). IL-12 has an anti-angiogenic effect in that it blocks the

formation of new blood vessels. It does this indirectly by increasing the levels of IP-10 via interferon gamma (Sgadari et al., 1996). The literature to explain the link between platelets and IL-12/IL-12p70 is limited. However, a recent publication reported that the presence of low-level IL-12 (representative of chronic inflammation) caused platelets to be hyperreactive and spread (Page et al., 2018).

sP-selectin is a marker of platelet activation and section 4.2.5 showed that sP-selectin correlates with duration of diabetes. Work from this chapter has identified significant positive correlations with sP-selectin and the inflammatory markers IL-1β, MCP-1 and IP-10. IL-1β is a proinflammatory cytokine synthesised from mRNA within activated platelets (Vieira de Abreu et al., 2013), and both IL-1β and MCP-1 are secreted by endothelial cells in the presence of activated platelets (Gawaz et al., 1998; Cha et al., 2000). IP-10 correlated with α-granule secretion in activated platelets. Given that IP-10 is a downstream molecule to IL-12 in angiogenesis inhibition (Sgadari et al., 1996), one could propose that an anti-angiogenic effect is initiated by platelet hyperactivation in a positive feedback mechanism. It is interesting to note that HbA1c was positively associated with IP-10. This, again, could be a feedback mechanism to reduce angiogenesis in the presence of high glucose.

It has been known since the 1990's that both resting and activated platelets could adhere to the vessel wall without vessel injury and recruit inflammatory cells (Frenette et al., 1995). Results from this study further our understanding of

platelets and inflammation by showing that both stimulated and non-stimulated platelets in type 2 diabetic patients are proinflammatory.

6 Comparison of Platelet activity in healthy, Hyperlipidaemic and Diabetic Participants

6.1 Introduction

Evidence suggests that type 2 diabetic patients have increased platelet reactivity compared to healthy subjects, such as elevated expression of surface activation markers (Keating et al., 2003; (Yngen et al., 2001). Conversely, Shlomai et al. (2015) reported no difference in P-selectin expression and $\alpha_{IIb}\beta_3$ activation between diabetic patients and a closely matched control group (Singer et al., 2014).

As hyperglycaemia is the cornerstone of diabetes, many studies have focused on the relationship between hyperglycaemia and platelet hyperreactivity, with differing results. For example, induction of hyperglycaemia in healthy groups has been shown to increase platelet activation and aggregation (De la Cruz et al., 2004; Sudic et al., 2006; Tang et al., 2011). Yet these results can vary depending on the conditions of the experiment e.g. incubation times, agonist used and the agonist concentration. Although hyperglycaemia is the *sine qua non* of diabetes, patients also exhibit altered lipid levels, and hyperlipidaemia has been reported to increase platelet reactivity (Chan et al., 2014). Thus, hyperglycaemia and/or hyperlipidaemia could increase platelet reactivity in subjects with type 2 diabetes.

The correlation data from section 4.2.6 identified that the most significant risk factor for platelet hyperreactivity in type 2 diabetic patients is LDL-C. This indicated that dyslipidaemia, not hyperglycaemia, is associated with platelet hyperreactivity in diabetes. To investigate this further, a pilot study was designed to measure biochemical parameters and platelet reactivity in three cohorts: control, hyperlipidaemic and type 2 diabetic patients. The control group consisted

of participants who were not treated for any health complications. The hyperlipidaemic group was chosen because the participants had similar baseline characteristics to the diabetic cohort (such as BMI and lipid profile) but did not have hyperglycaemia. It was hypothesised that the control group would exhibit reduced platelet reactivity compared to the other groups, and the hyperlipidaemic and diabetic groups would exhibit similar platelet activation due to having similar lipid levels.

To compare platelet reactivity between the three groups, measurements of platelet indices, IPF, platelet activation and aggregation was undertaken.

6.2 Results

6.2.1 Baseline Characteristics for the Participants

Forty-five participants were recruited for this small pilot study to investigate platelet reactivity in patients with similar LDL-C levels, with and without diabetes. Fifteen hyperlipidaemic patients, were recruited from the Lipids Clinic at the Manchester Royal Infirmary (MRI), fifteen diabetic patients were recruited from the Diabetic Clinic (MRI) and a control group of fifteen participants who did not have diabetes or hyperlipidaemia were recruited. The three groups were matched as closely as possible for age, male/female ratio and BMI (Table 6.1). The BMI for the diabetic cohort, however, was significantly higher than the control group (Tukey, $p=0.01$), and age was significantly lower in the control group. No participant from the control group had a BMI over 30 kg/m^2, and, thus, not categorised as obese (World Health Organisation, 2017). In contrast, three (21.0%) of hyperlipidaemic patients and eight (53.3%) diabetic patients were obese.

All hyperlipidaemia and diabetic patients recruited to the study were taking lipid lowering medication (statins). Two (13.3%) hyperlipidaemic and ten (66.6%) diabetic patients were also on anti-hypertensive drugs. The control group were not on any medication for hypertension, hyperlipidaemia or diabetes. There were no current smokers in the control group, four (26.6%) in the hyperlipidaemic group and two (13.3%) in the diabetic group.

As expected, the mean FBG and HbA1c was similar for the healthy and hyperlipidaemic, and significantly increased in the diabetic group (Table 6.1). There was no significant difference between all three groups for the total cholesterol, HDL-C and triglycerides. Contrary to what was expected, the control group had significantly higher LDL-C levels than the hyperlipidaemic and diabetic groups ($p=0.04$ and $p=0.02$, respectively). It is likely that the lipid lowering medication was causing this significant reduction in LDL-C levels in the hyperlipidaemic and diabetic groups. The serum creatinine was considerably out of range for the males in the diabetic group (n=3) over the recommended level of 104 µmol/L), and moderately out of range for one of the hyperlipidaemic males, suggesting that kidney injury was present within these two cohorts. However, the means are within the recommended creatinine levels for all three cohorts.

Table 6.1: Baseline characteristics for the control, hyperlipidaemic and diabetic participants

	Control n=15	**Hyperlip-idaemic n=15**	**Diabetic n=15**	**P value control vs. lipid**	**P value control vs. diabetic**	**P value lipid vs. diabetic**
M/F (*n*)	7/8	7/8	6/9	----	----	----
Age (yr), mean ± SD	45.9 ± 10.2	55.1 ± 10.7	62.9 ± 6.8	**0.010****	**<0.0001 ****	0.064
Age (yr), range	32-65	27-75	52-71	----	----	----
Duration of diabetes (yr), mean ± SD	0.0	0.0	12.6 ± 4.9	----	----	----
BMI (kg/m^2), mean ± SD	25.8 + 3.7	27.0 ± 3.5	30.8 ± 5.9	0.764	**0.012****	0.082
Obesity (BMI >30) (%)	0.0	21.0	53.3	----	----	----
Current smoker (%)	0.0	26.6	13.3	----	----	----
Previous smoker (%)	13.3	0.0	6.6	----	----	----
Medication Profile (%)						
Insulin	0.0	0.0	46.6	----	----	----
Other anti-hyperglycaemic medication	0.0	0.0	60.0	----	----	----
Antihypertensive agents	0.0	13.3	66.6	----	----	----
Lipid lowering drugs (incl. statins)	0.0s	100.0	100.0	----	----	----
History of (%)						
Peripheral neuropathy	0.0	0.0	20.0	----	----	----

Table 6.1 cont

	Control n=15	Hyperlip-idaemic n=15	Diabetic n=15	P value control vs. lipid	P value control vs. diabetic	P value lipid vs. diabetic
History of (%)						
Retinopathy	0.0	0.0	26.6	----	----	----
Acute kidney injury	0.0	0.0	0.0	----	----	----
Hypertension	0.0	27.3	50.0	----	----	----
Laboratory variables						
FBG (mmol/L), mean ± SD	4.9 ± 0.5	4.9 ± 0.7	9.6 ± 4.1	>0.999	**0.008****	**0.009****
HbA1c (mmol/mol), mean ± SD	33.4 ± 3.3	36.9 ± 3.0	66.8 ± 20.7	0.67	**<0.0001 ******	**<0.0001 ******
Total Cholesterol (mmol/L), mean ± SD	5.2 ± 0.9	4.7 ± 1.1	4.8 ± 1.2	0.50	0.68	0.95
LDL-C (mmol/L), mean ± SD	3.2 ± 0.8	2.4 ± 1.1	2.3 ± 0.8	**0.04***	**0.02***	>0.99
HDL-C (mmol/L), mean ± SD	1.3 ± 0.3	1.6 ± 0.4	1.5 ± 0.5	0.35	0.57	0.92
Triglycerides (mmol/L), mean ± SD	1.5 ± 0.6	1.6 ± 1.0	2.0 ± 0.9	>0.99	0.39	0.39
Serum creatinine (µmol/L), range (min-max)	Female: 59-74, Male: 61-109	Female: 58-81, Male: 67-142	Female: 51-82, Male: 63-190	----	----	----

Table 6.1 cont.

	Control n=15	Hyperlip-idaemic n=15	Diabetic n=15	P value control vs. lipid	P value control vs. diabetic	P value lipid vs. diabetic
Serum creatinine, mean ± SD	78.8 ± 15.3	78.6 ± 20.6	84.5 ± 38.4	>0.99	>0.99	>0.99

All p-values calculated using the Tukey's multiple comparisons test for parametric data, except data for the variables FBG, LDL-C, VLDL-C, triglycerides and serum creatinine, which were calculated using the Dunn's test for non-parametric data. P values ≤ 0.05 represented as *. P values ≤ 0.01 represented as **. P values ≤ 0.001 represented as ***. P ≤ 0.0001 represented as ****.

6.2.2 Platelet Indices

Elevated MPV has been reported to be associated hyperglycaemia (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder et al, 2014; Ulutas et al., 2014) and data from this study has shown that platelet count is linked with obesity (Table 4.3). Comparisons were made for platelet indices in the three cohorts (Table 6.2).

The hyperlipidaemic and diabetic groups had lower platelet counts compared to the control group, a one-way ANOVA showed no significant difference between all three groups ($p=0.430$) and they were all within the normal reference range. In addition, there was no significant difference in the MPV, PDW or P-LCR for all three groups (one-way ANOVA, $p=0.595$, $p=0.397$ and $p=0.509$, respectively), nonetheless the diabetic group did have the highest MPV, PDW and P-LCR. The modest sample size in the present study ($n=15$) may have contributed to the limiting significance of the two platelet parameters.

Table 6.2: Mean platelet count and MPV for control, hyperlipidaemic and diabetic participants

	Control n=15	Lipid n=15	Diabetic n=15	P value control vs. lipid	P value control vs. diabetic	P value lipid vs. diabetic
Platelet count ($x10^9$), mean ± SD	286.5 ± 80.8	257.0 ± 71.9	257.3 ± 57.0	0.632	0.468	>0.999
MPV (fL), mean ± SD	10.8 ± 1.2	10.8 ± 0.7	11.1 ± 0.9	0.995	0.683	0.624
PDW (fL), mean ± SD	13.08 ± 2.42	12.95 ± 1.60	13.92 ± 2.18	0.982	0.526	0.419
P-LCR (%), mean ± SD	32.21 ± 9.58	31.73 ± 6.17	34.89 ± 7.77	0.985	0.630	0.528

P-values for the MPV calculated using the Tukey's multiple comparisons test for parametric data. P values for the platelet count calculated using the Dunn's test for non-parametric data.

6.2.3 Thrombopoiesis

High levels of reticulated platelets are associated with elevated thrombopoiesis and Mijovic et al. (2015) reported that reticulated platelets were increased in diabetic patients compared to control subjects. Moreover, hyperlipidaemic mice had increased platelet turnover and clearance from the circulation, suggesting that cholesterol metabolism effects turnover (Dole et al., 2008).

To investigate whether thrombopoiesis was altered between the three groups, the percentage of reticulated platelets was measured as an indication of IPF. A Kruskal-Wallis test showed a significant increase in the % of reticulated platelets in the control group compared to the hyperlipidaemic group ($p=0.032$) (Table 6.3 and Figure 6.1). Given that the control group had the highest LDL-C levels (Table 6.1) this data was consistent with data from the diabetic cohort (Chapter 4), which showed a positive correlation between percentage of reticulated platelets and LDL-C (Figure 4.4). To determine whether the relationship between LDL-C and reticulated platelets was present across the three groups and independent of diabetes, a Spearman correlation was performed using the combined data for the 3 groups ($n=45$). There was a significant correlation with LDL-C levels when the 3 cohorts were combined ($r=0.373$, $p=0.016$, $n=45$) (Figure 6.2A).

The forty-five participants were separated into three groups according to LDL-C level ($<\underline{2}.0$, 2.1-2.9 and $>\underline{3}.0$ mmol/L). A Kruskal-Wallis test showed a significant difference between the groups ($p=0.009$) (Table 6.4). A Dunn's multiple comparisons test showed a significant increase in the % of reticulated platelets for participants with LDL-C $\geq$3.0 mmol/L compared to participants with LDL-C

≤2.0 mmol/L (p=0.008) (Figure 6.2B). No significant difference was observed with ≤2.0 vs. 2.1-2.9 mmol/L (p=>0.999) and with ≥3.0 mmol/L vs. 2.1-2.9 mmol/L (p=0.123).

Table 6.3: Mean % of reticulated platelets for control, hyperlipidaemic and diabetic participants

	Control n=15	Lipid n=15	Diabetic n=15	P value control vs. lipid	P value control vs. diabetic	P value lipid vs. diabetic
Reticulated platelets (%), mean ± SD	11.57 ± 5.82	8.46 ± 7.23	10.31 ± 5.02	0.032*	>0.999	0.481

P values calculated using the Dunn's test for non-parametric data. * represents p ≤0.05.

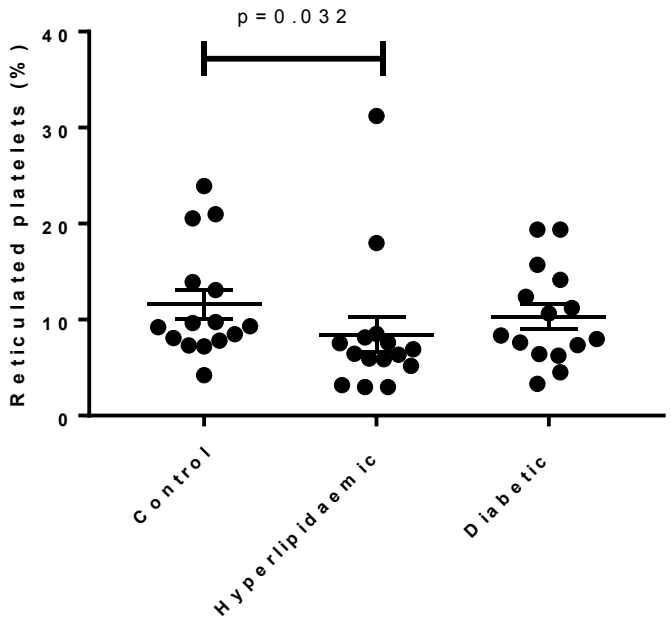

Figure 6.1: Mean values of reticulated platelets in control, hyperlipidaemic and diabetic patients
A Kruskal-Wallis test showed a significant increase in the % of reticulated platelets for the control group compared to the hyperlipidaemic group (p=0.032, n=15 per group). Bars represented as mean ± SEM.

Table 6.4: The comparison between LDL-C levels and reticulated platelets

	LDL-C ≤2.0 mmol/L n=17	LDL-C 2.1-2.9 mmol/L n=14	LDL-C ≥3.0 mmol/L n=14	P value ≤2.0 vs. 2.1-2.9 mmol/L	P value ≤2.0 vs. >3.0 mmol/L	P value 2.1-2.9 vs. ≥3.0 mmol/L
Reticulated platelets (%), mean ± SD	7.99 ± 4.32	8.57 ± 4.35	14.24 ± 7.55	>0.999	**0.008****	0.123

P values calculated using the Dunn's multiple comparisons test for non-parametric data. ** represents p ≤0.01.

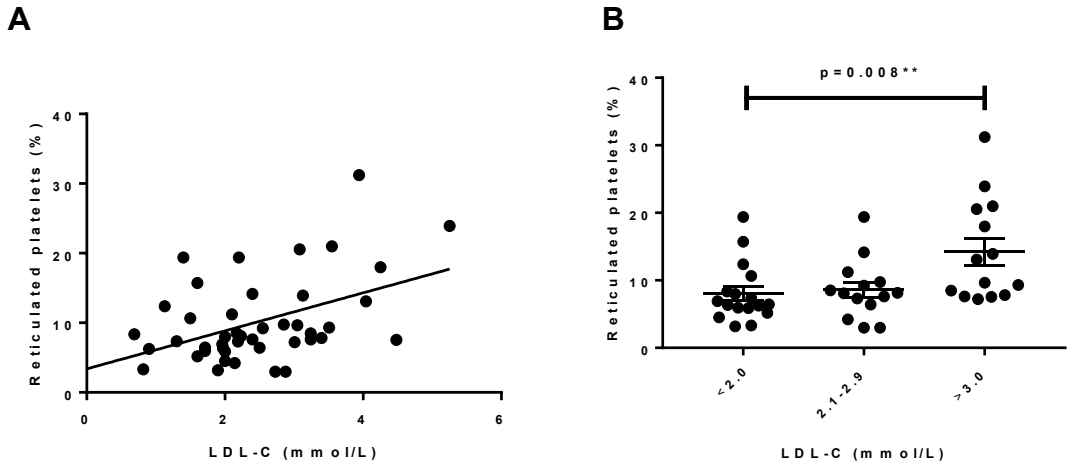

Figure 6.2: Correlation between reticulated platelets and LDL-C

Figure A: Spearman test for non-parametric data shows a significant positive correlation for reticulated platelets and LDL-C (r=0.373, P=0.016) (n=45). **Figure B:** The reticulated platelets (%) in three groups separated by LDL-C levels: below 2.0, 2.0 – 3.0 and above 3.0 (n=17,14 and 14, respectively). A Kruskal-Wallis test showed a significant difference in the % of reticulated platelets for the three groups (p=0.004). A Dunn's multiple comparisons test showed a significant increase with ≥3.0 mmol/L compared to ≤2.0 mmol/L. Bars represented as mean ± SEM.

6.2.4 Platelet Activation Markers

Elevated expression of surface activation markers is associated with diabetes (Keating et al., 2003; Yngen et al., 2001) and hyperlipidaemia (Pawelczyk et al., 2009). In this study, the levels of platelet activation markers CD62P and activated $\alpha_{IIb}\beta_3$ on non-stimulated platelets were measured in the three cohorts to assess the activation status of circulating platelets. The α-granule secretion, indicated by CD62P surface expression was increased in the hyperlipidaemic and diabetic participants, however this was not statistically significant (Table 6.5). The PAC-1 binding was very similar in all three groups (p=>0.9 for each Tukey multiple comparison test.

Agonist induced α-granule secretion was significantly increased in platelets from diabetic patients compared to control subjects (p=0.002) (Table 6.6 and Figure 6.3B). No significant difference was observed for PAC-1 binding in activated platelets for the three groups.

Table 6.5: Mean MFI for activation markers in resting platelets from control, hyperlipidaemic and diabetic participants

	Control n=15	Lipid n=15	Diabetic n=15	P value control vs. lipid	P value control vs. diabetic	P value lipid vs. diabetic
CD62P expression (MFI), mean ± SD	15.5 ± 9.4	26.3 ± 25.8	21.3 ± 14.2	0.861	0.654	>0.999
PAC-1 binding (MFI), mean ± SD	10.9 ± 8.7	10.3 ± 6.2	10.7 ± 4.0	0.966	0.998	0.979
CD62P expression (MFI), mean ± SD	952.1 ± 396.1	1268.5 ± 381.7	1515.1 ± 510.8	0.125	**0.002****	0.275
PAC-1 binding (MFI), mean ± SD	286.5 ± 103.5	271.7 ± 71.6	302 ± 77.7	0.885	0.873	0.599

All p-values calculated using the Tukey's multiple comparisons test for parametric data except CD62P expression in resting platelets.

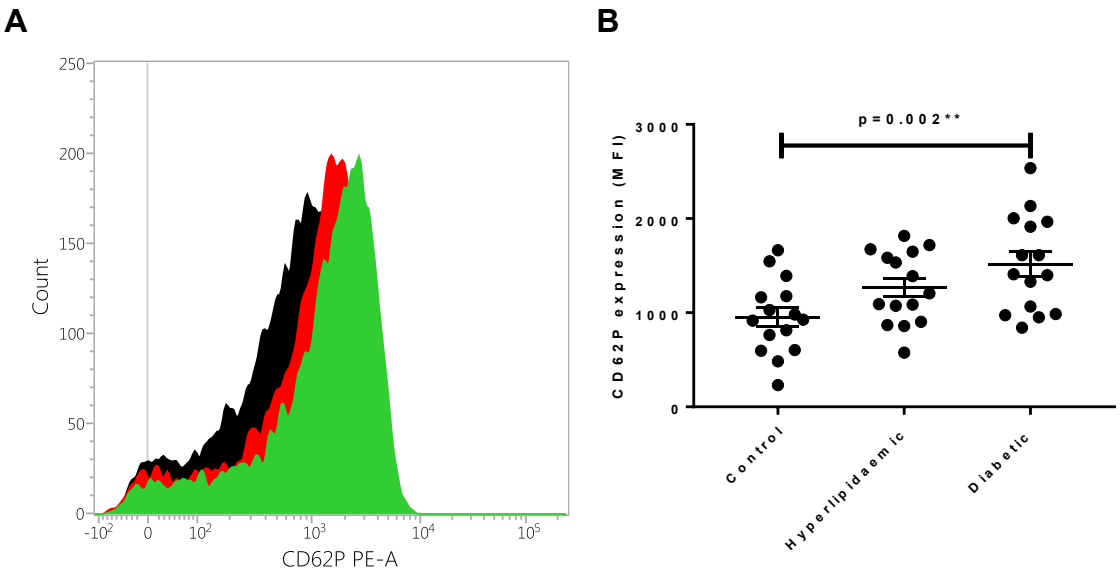

Figure 6.3: α-granule secretion in ADP-activated platelets from control, hyperlipidaemic and diabetic participants

Figure A: A histogram to show α-granule secretion in one control (black), hyperlipidaemic (red) and diabetic (green) participant. The CD62P MFI for each participant is 814, 1389 and 1965, respectively. **Figure B:** A one-way ANOVA showed a significant difference in α-granule secretion (CD62P membrane expression) in ADP-activated platelets between the three groups (p=0.004). A Tukey's multiple comparison test showed a significant increase in α-granule secretion in type 2 diabetic patients compared to the control group (p = 0.002) and no difference between control vs. hyperlipidaemic and diabetic vs. hyperlipidaemic (p=0.125 and p=0.275 respectively). Bars represented as mean ± SEM (n=15 per group).

The significant increase in α-granule secretion observed in the diabetic group compared to the control group indicates that hyperglycaemia may be responsible for the increased platelet reactivity to ADP, since the diabetic group had a significantly higher FBG and HbA1c (Table 6.2). This is inconsistent however with data in Chapter 4, where there was no relationship between hyperglycaemia and platelets reactivity. Other characteristics that were significantly different between the diabetic and control group were age and BMI. Correlations were therefore performed across all three groups to investigate whether the enhanced α-granule secretion observed in the diabetic group was related to the increased age or BMI of the group. It was determined that age significantly correlated with CD62P

activation (p=0.002), indicating that this was the reason for the enhanced α-granule secretion in the diabetic cohort.

Table 6.6: Correlation between ADP-induced CD62P vs. BMI and age

	CD62P in resting platelets n=45	
	R	*P*
Age	**0.456**	**0.002****
BMI	<0.001	0.998

P values calculated using a Pearson test. ** represents p ≤0.01.

6.2.5 Platelet Aggregation

Increased platelet aggregation is associated with hyperglycaemia (De la Cruz et al., 2004; Sudic et al., 2006; Tang et al., 2011) and hyperlipidaemia (Chain et al., 2014). To investigate whether platelet aggregation was altered between the three groups, PRP was stimulated with collagen (1 µg/ml) or ADP (5 µmol/L) and maximal aggregation measured after 5 minutes. The lag time was also assessed to determine whether the initial rate of aggregation was altered. The diabetic and healthy cohorts had similar maximal ADP-activated aggregation (79.2 ± 9.6% and 76 ± 19.0% respectively, p=<0.999; Figure 6.4A) with similar lag times (15.3 ± 6.4 sec and 12 ± 3.1 sec, respectively, p=0.169; Figure 6.4B). The hyperlipidaemic patients had reduced ADP-activated aggregation, although this was not significant. The lag time (19.7 ± 8.8 sec) however was significantly longer than the control group (13 ± 3.1 sec; p=0.002).

Collagen-activated aggregation was increased in control participants (87.5 ± 4.4%) compared to hyperlipidaemic participants (79.8 ± 9%; p=0.025), but no significant difference was shown with the diabetic cohort (Figure 6.4C). The lag time for collagen induced-aggregation was also significantly lower in the control group (45.5 ± 17.4s) compared to the hyperlipidaemic (70.4 ± 16.2s; p=0.0004) and the diabetic group (67.53 ± 15.5, p=0.002) (Figure 6.4D).

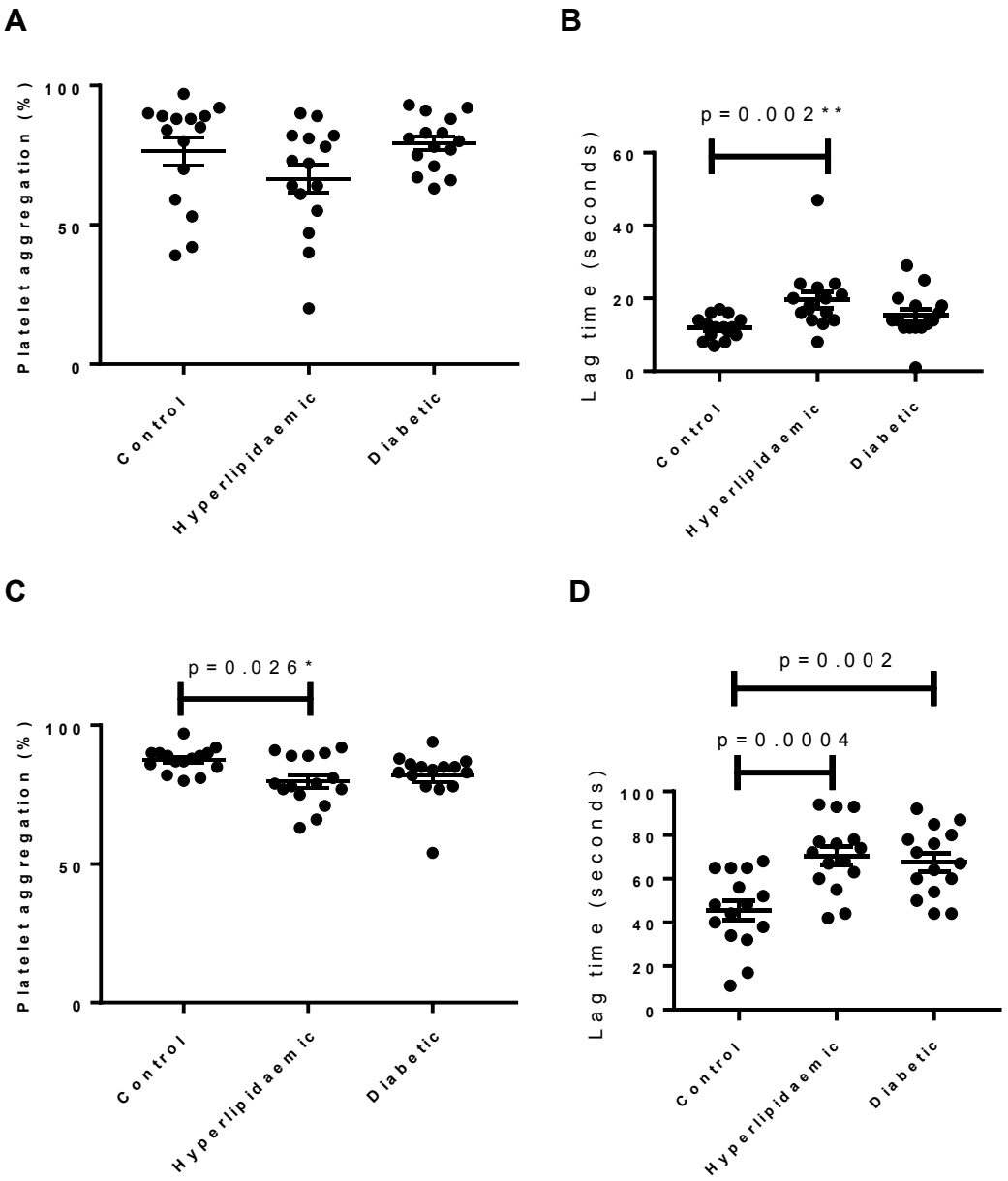

Figure 6.4: Platelet aggregation and lag time in control, hyperlipidaemic and diabetic participants

Figure A: There was no statistical difference in ADP-activated maximal aggregation between the three groups. **Figure B:** There was significantly longer lag time for hyperlipidaemic participants compared to control participants (p=0.002, Dunn's test). **Figure C:** There was a significant increase in collagen-activated platelet aggregation compared to control participants (p=0.026, Kruskal Wallis test). **Figure D:** There was a strong significant increase in lag-time for hyperlipidaemic participants compared to the control group (p=0.0004, Tukey test) and control compared to the diabetic group (p=0.002, Tukey). Bars represented as mean ± SEM (n=15 per group).

6.2.6 LDL-C and Platelet Aggregation

Platelet aggregation assays indicate that the control group, which has significantly higher LDL-C levels than both the hyperlipidaemic and diabetic groups (Table 6.2), also presents with more responsive platelets. To determine whether there was a relationship between LDL-C and platelet aggregation, comparisons were carried out for collagen and ADP-induced aggregation/lag time responses against LDL-C levels ($\leq$2.0, 2.1-2.9 and $\geq$3.0 mmol/L).

The data demonstrated that there were no significant differences between the LDL-C levels for ADP and collagen maximal % aggregation (Table 6.7 and Figure 6.5A and C). There was no significant difference between $\leq$2.0 and $\geq$3.0 mmol/L for ADP-stimulated lag, and a significant increase in lag-time for $\leq$2.0 for collagen-stimulated lag-time compared to $\geq$3.0 mmol/L ($p=0.024$) (Table 6.7 and Figure 6.5B and D).

Table 6.7: Mean platelet aggregation (%) for different LDL-C levels

	≤2.0 mmol/L LDL-C	**2.1-2.9 mmol/L LDL-C**	**≥3.0 mmol/L LDL-C**	**P value ≤2.0 vs. 2.1-2.9**	**P value ≤2.0 vs. ≥3.0**	**P value 2.1-2.9 vs. ≥3.0**
ADP-stimulated aggregation (%)	70.12 ± 18.5	79.63 ± 7.23	11.76 ± 18.6	0.444	>0.999	>0.999
ADP-stimulated lag time (sec)	17.12 ± 7.03	14.08 ± 4.25	15.41 ± 8.75	0.797	0.142	>0.999
Collagen-stimulated aggregation (%)	81.12 ± 7.12	82.6 ± 10.46	85.23 ± 7.43	0.331	0.342	>0.999
Collagen-stimulated lag time (sec)	72.33 ± 16.36	57.17 ± 22.29	54.47 ± 17.15	0.097	**0.024***	0.921

All p values calculated using the Kruskal Wallis test for non-parametric data except the comparisons with collagen-stimulated lag time. Data represented as mean ± SD.

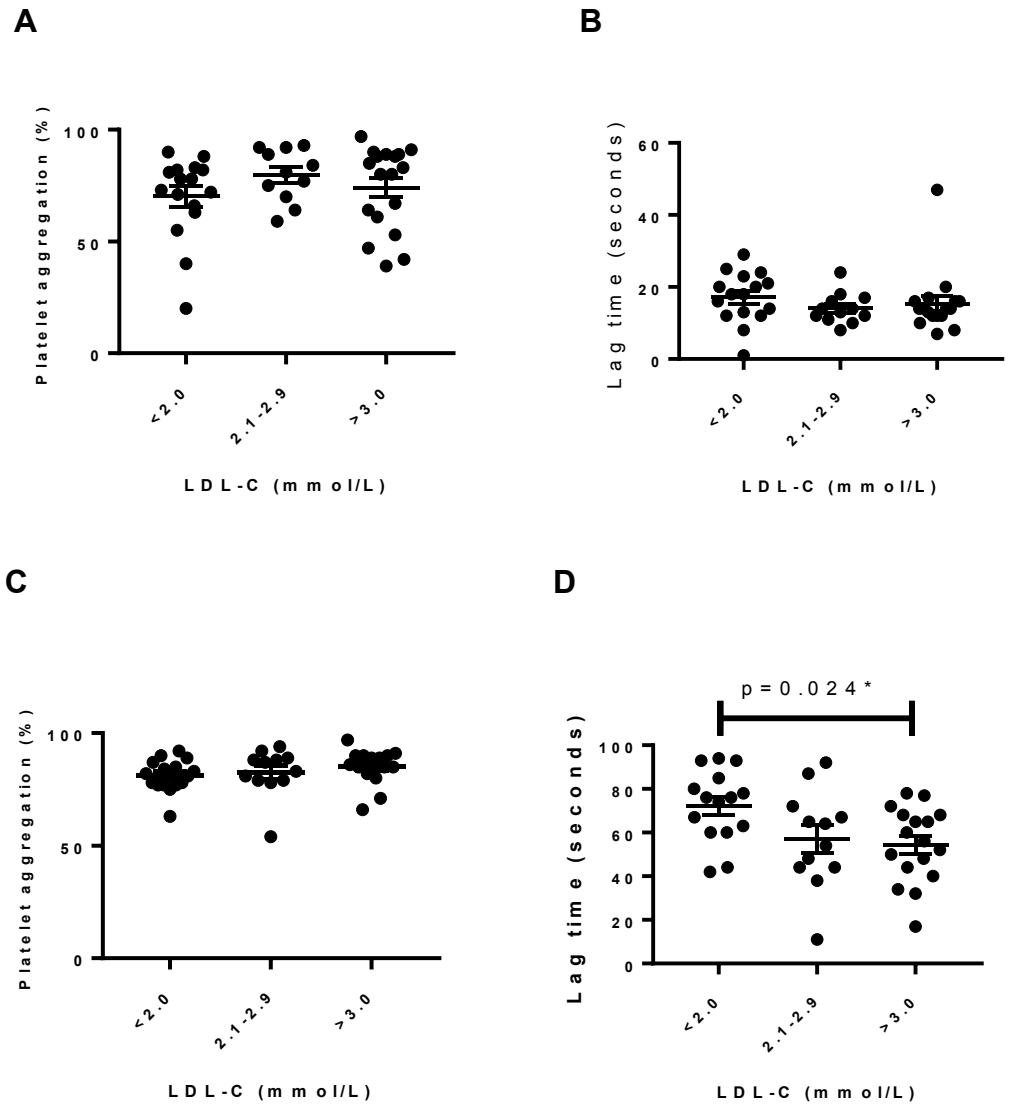

Figure 6.5: Comparisons between LDL-C levels and platelet aggregation
Figure A: LDL-C levels do not have significantly different ADP-stimulated platelet aggregation. **Figure B:** LDL-C levels do not have significantly different ADP-stimulated lag-time. **Figure C:** LDL-C levels do not have significantly different collagen-stimulated platelet aggregation. **Figure D:** Participants with LDL-C levels <2.0 mmol/L have significantly increased lag-time compared to those with >3.0 mmol/L (Tukey, p=0.024) (n=16, 12, 17, respectively). Bars represented as mean ± SEM.

6.3 Discussion

Many studies have focused on hyperglycaemia as a proposed risk factor for platelet hyperreactivity in type 2 diabetes. Yet diabetes patients also exhibit characteristics of dyslipidaemia such as elevated LDL-C, decreased HDL-C and increased triglycerides (Bell et al., 2011), and hyperlipidaemia is associated with elevated platelet activation (Chan et al., 2014).

The study aimed to further our understanding of the risk factors associated with diabetes, by investigating whether hyperglycaemia and/or hyperlipidaemia is involved in altered platelet function. This was attained by comparing the lipid profile and platelet reactivity in control, hyperlipidaemic and type 2 diabetic patients. Participants were matched as closely as possible for age, sex and BMI since these have all been demonstrated to affect platelet function responses (Gleerup and Winther, 1995; Cowman et al., 2015; Schneider et al., 2009b).

The data from earlier chapters has shown that hyperglycaemia is not associated with platelet reactivity, and that LDL-C correlates significantly with platelet aggregation. Consequently, we hypothesised that the hyperlipidaemic and diabetic groups would both have elevated platelet reactivity due to altered lipid levels in the blood milieu.

Unexpectedly, measurement of the baseline cholesterol levels identified that the control group had increased LDL-C levels compared to the hyperlipidaemic and diabetic cohorts. This observation ascertained that the statin treatment was

having a significant effect on LDL-C levels in the hyperlipidaemic and diabetic groups.

Findings from sections 3.2.1 and 4.2.2 showed that high glucose was associated with increased MPV, and that this may be through osmotic stress associated with uptake of glucose via GLUT transporters (Lösche et al., 1989). In this study there was a trend towards a large MPV in the diabetic cohort, however the smaller sample size did not allow it to reach significance. Previous studies that have found a significant difference used a large sample size (Demirtunc et al., 2009; Kodiatte et al., 2012; Ozder et al, 2014; Ulutas et al., 2014).

The control group had an elevated % of reticulated platelets compared to the hyperlipidaemic group. Additionally, the IPF correlated with LDL-C across the three groups, and subjects with LDL-C over 3.0 mmol/L had significantly elevated IPF compared to LDL-C under 2.0 mmol/L. Previous studies have linked plasma lipid levels with platelet biogenesis and /or turnover. For example, Fessler et al. (2013) reported a correlation between platelet count and non-HDL in 10,000 participants. Unesterified cholesterol accumulation in the platelet membranes of hyperlipidaemic mice led to increased platelet turnover and clearance from the circulation to induce thrombocytopenia (Dole et al., 2008). Our study, however, did not show any difference in platelet count, indicting no effect on platelet destruction. To the best of our knowledge, this is the first study to associate elevated levels of LDL-C with IPF, and further work is required to investigate the mechanisms that contribute to increased platelet production in the presence of high LDL-C.

Studies have reported an association between platelet activation markers with diabetes (Keating et al., 2003; Yngen et al., 2001) and hyperlipidaemia (Pawelczyk et al., 2009). Although the CD62P expression and PAC-1 binding was increased in resting platelets for the hyperlipidaemic and diabetic groups, this increase was not significant. Also, no significance was shown for PAC-1 binding in both resting and ADP-activated platelets for the three cohorts. A significant increase in CD62P expression in ADP-activated platelets was found to be associated with age. Shlomai et al. (2015) reported no difference in platelet expression markers between diabetic and non-diabetic groups without prior ischemic events. However, the diabetic subjects used for the study had well-controlled glycaemia. The diabetic subjects in our study had significantly elevated HbA1c, representing a group with poorly controlled glycaemia.

The platelet aggregation was increased in the control group compared to the hyperlipidaemic group and diabetic group. Findings from section 6.2.3 showed an association between LDL-C and the % of reticulated platelets, independent of diabetes. Previous *in vitro* work has shown increased aggregating potential in reticulated platelets, that formed the core of aggregates to recruit older platelets (Armstrong et al., 2017).

The data showed that there was a significant increase in lag-time for collagen-stimulated aggregation in participants with LDL-C <2.0 mmol/L compared to those with >3.0 mmol/L. This demonstrates that the effect of LDL-C on platelet aggregation is independent of diabetes. This potentiation could be caused by cholesterol accumulation in the platelet membranes which disturbs the

membrane structure, and enhances platelet receptor signalling, as shown in various hematopoietic cells (Zhu et al., 2010; Yvan-Charvet et al, 2010).

It is important to note that the hyperlipidaemic group had significantly decreased platelet aggregation compared to the control group, but the diabetic group did not. It has been reported that statin treatment can reduce platelet aggregation in hyperlipidaemic patients (Sikora et al., 2013). Potentially, diabetic patients have additional complications (possibly associated with hyperglycaemia) that alters the beneficial effects statin treatment has on reducing platelet function, although these mechanisms remain unclear. Further studies to compare the effect of statins on platelet function in diabetic and non-diabetic subjects would be beneficial to further our understanding of platelet hyperreactivity in statin-treated diabetic patients.

In conclusion, this study has identified a link between the % of immature platelets and LDL-C, that is independent of diabetes. Understanding the mechanistic processes that contribute to the regulation of platelet production and activation by cholesterol could identify new therapeutics for atherothrombosis. Additionally, statin therapy can reduce platelet reactivity, however, they are not as effective at reducing platelet aggregation in type 2 diabetic patients.

7 Key findings and Clinical Outcomes

7.1 Overall Discussion

Atherothrombosis is the leading cause of death in type 2 diabetic patients (Reusch and Drazin, 2007) and they are at equal risk of myocardial infarction as non-diabetic subjects with a prior myocardial infarction (Haffner et al., 1998; Schramm et al., 2008). Despite this, the current NICE guidelines are to offer antiplatelet therapy (such as aspirin or clopidogrel) for adults with type 2 diabetes as a secondary prevention of cardiovascular disease (NICE, 2018). Platelet hyperreactivity and reduced sensitivity to standard antiplatelet agents has been repeatedly reported in the literature (Mehta et al., 2000; Ogawa et al., 2009; Belch et al., 2008).

Enhanced platelet reactivity may contribute to the development of atherosclerosis and plaque progression, but also the development of atherothrombosis and the severity of the thrombotic response. This could potentially make the difference between complete artery occlusion and a fatal myocardial infarction and partial occlusion and survival.

Despite numerous studies investigating the mechanism that underpin platelet reactivity, there is no clear consensus and the process remains unclear. Here we present data that challenges the current paradigm and demonstrates no role for hyperglycaemia in enhancing platelet reactivity in type 2 diabetes. It should be taken into consideration that less recent studies reporting an association between hyperglycaemia and platelet reactivity were using a diabetic cohort that were not taking lipid lowering medication, therefore any effects of hyperglycaemia may be dependent on higher LDL-C levels.

We identified platelets as proinflammatory mediators in type 2 diabetic patients, supporting other literature in this field of study (Frenette et al., 1995; Massberg et al., 2002). The positive correlations with inflammatory mediators and both sP-selectin and P-selectin surface expression in resting platelets (section 5.2.3) indicated that type 2 diabetic patients with circulating platelets in a more active state have higher levels of inflammation. This would suggest that the combination of anti-platelet therapy with anti-inflammatory medication may improve anti-thrombotic therapies in type 2 diabetic patients. Though it is not clear whether the inflammation causes the expression of activation markers on circulating platelets or whether activated platelets stimulate the inflammatory mediators. Enhanced adhesion receptor expression and inflammation may contribute to atherogenesis and plaque progression. The data however demonstrated that the level of inflammatory molecules in the plasma did not relate to the responsiveness of platelets to standard agonists.

The most significant finding from this thesis is that LDL-C may be used as a potential biomarker for thrombotic risk in type 2 diabetic patients. LDL-C potentiates platelet aggregation in type 2 diabetic patients. Most importantly, patients with LDL-C above 2.0 mmol/L have significantly increased platelet aggregation. This potentiation is possibly caused by native LDL-C, not ox-LDL, and is independent of the diabetic condition. Cholesterol accumulation in the platelet membranes may disturb membrane structures, particularly lipid rafts, and enhance platelet receptor signalling, as shown in various hematopoietic cells (Zhu et al., 2010; Yvan-Charvet et al, 2010). Thus, plasma membrane cholesterol accumulation in platelets could potentially alter signalling via surface receptors,

such as $P2Y_{12}$. This may lead to the increased receptor expression found in diabetic platelets (Hu et al., 2017). Moreover, we identified that LDL-C is associated with thrombopoiesis. Newly released platelets are more reactive, with greater prothrombotic potential (Mijovic et al., 2015), and younger platelets form the core of aggregates to recruit older platelets (Armstrong et al., 2017). Furthermore, diabetic patients do not respond well to antiplatelet therapy such as aspirin, and it is thought that this is due to the high levels of immature platelets because the newly released platelets have not been inhibited by the aspirin (Guthikonda et al., 2007).

Together these findings indicate that high LDL-C levels in type 2 diabetic patients causes the accumulation of cholesterol in platelets. This elevates platelet production and increases reactivity which may contribute to atherothrombosis. The combination of antiplatelet therapy and lipid lowering treatment may reduce platelet production and hyperreactivity in type 2 diabetic patients with LDL-C above 2.0 mmol/L.

7.2 Summary of key findings from the whole study

1. Hyperglycaemia enhances MPV in circulating platelets through osmosis.
2. Hyperglycaemia does not increase platelet reactivity as previously reported.
3. Enhanced activation of circulating platelets is accompanied by elevated inflammatory mediators.
4. The most significant risk factor for platelet reactivity in type 2 diabetic patients is native LDL-C.
5. LDL-C levels >2.0 mmol/L significantly increases platelet aggregation in type 2 diabetic patients.
6. LDL-C is associated with elevated platelet production, independent of diabetes.
7. LDL-C >3.0 mmol/L significantly increases platelet production compared to <2.0 mmol/L.

7.3 Clinical Significance

This demonstrates that LDL-C may act as a biomarker to identify type 2 diabetes patients who would benefit from antiplatelet prophylaxis. Specifically, patients with LDL-C >2.0 mmol/L will benefit most from antiplatelet therapy as a primary prevention for cardiovascular disease. Additionally, lipid lowering medication indirectly provides antithrombotic benefit by reducing LDL-C levels. Moreover, enhanced thrombopoiesis mediated by elevated LDL-C may be responsible for reduced antiplatelet efficacy in type 2 diabetic patients.

7.4 Limitations

1. A large number of patients were injecting insulin. This effected our ability to study the effect of insulin concentration on platelet reactivity, as patients would have elevated levels of insulin at certain times in the day due to the insulin treatment.
2. While this study was designed to have a stringent exclusion criterion to eliminate patients on drug treatments that would affect platelet function (e.g. anticoagulants, antiplatelet therapy), patients were also taking other medications that could affect the results, (i.e. glucose-lowering treatments such as Metformin).
3. The lack of standardisation associated with platelet function tests can affect the accuracy of the results. For example, preanalytical conditions (e.g. platelet count, lipid plasma, type of anticoagulant, haemolysis) and different procedural conditions (e.g. agonist concentration, PRP preparation) (Paniccia et al., 2015).
4. Multiplex immunoassays allow the measurement of multiple analytes in a single 25–50 µl sample which is really useful for studying cytokine responses. However, such multiplex approaches may compromise the ability of these assays to accurately measure actual cytokine levels (Djoba
5. Siawaya et al., 2008). Also, only one kit could be used in this study due to cost constraints, so sample replicates were unable to be used to improve accuracy of the results.
6. Not all assays were performed on all blood samples due to the following reasons a) amount of plasma collected from the patient during

venepuncture b) equipment failures on the day of blood collection c) reagent shortages.

7.5 Further work

To support the results from this study, it would be useful to focus on investigating the effects that LDL-C has on platelet activation and aggregation. For example, *in vitro* platelet function assays could be performed to determine whether platelet activity is increased in the presence of native-LDL and/or glycated-LDL. Also, further investigation into the mechanisms by which LDL promotes thrombopoiesis and enhances platelet reactivity. This could include using a megakaryocyte cell line (MEG-01 cells) to investigate whether LDL alters thrombopoietin signalling. It would also be useful to investigate platelets from patients with high and low LDL levels, and perform lipidomics and proteomics on the platelets to determine whether plasma LDL levels alter the membrane lipid constituents, and the lipid bases signalling molecules in platelets, or the levels of agonist receptors, particularly those which reside in lipid rafts e.g. $P2Y_{12}$. Lastly, a clinical study to compare the platelet reactivity in statin- and non-statin-treated diabetic patients, to further investigate the efficacy of lipid lowering treatment in the diabetic sub-population.

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9 Appendix

9.1 Dose responses

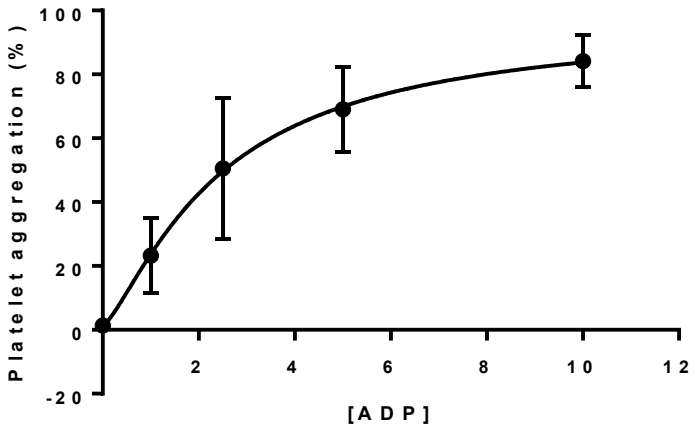

Figure 9.1: Dose response for the ADP in healthy PRP
Maximal platelet aggregation (%) for PRP stimulated with increasing ADP concentrations (µmol/L) in healthy participants. Curves were fitted by non-linear regression. The EC_{50} was 2.445 µmol/L. Data presented as mean ± SEM (n=8).

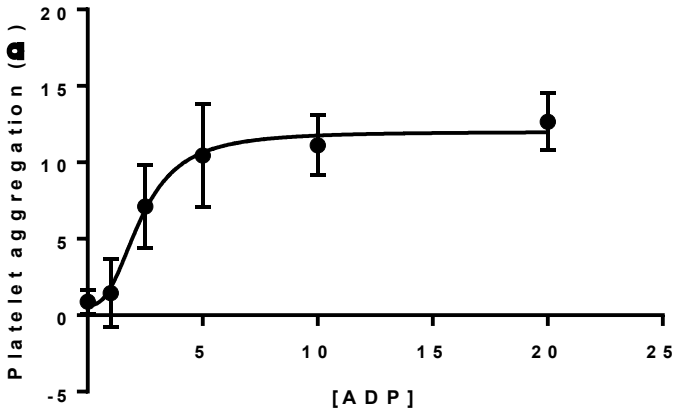

Figure 9.2: Dose response for ADP in healthy whole blood
Maximal platelet aggregation (Ω) for whole blood stimulated with increasing ADP concentrations in healthy participants. The EC_{50} was 2.33 µmol/L. Data represented as mean ± SEM (n=9).

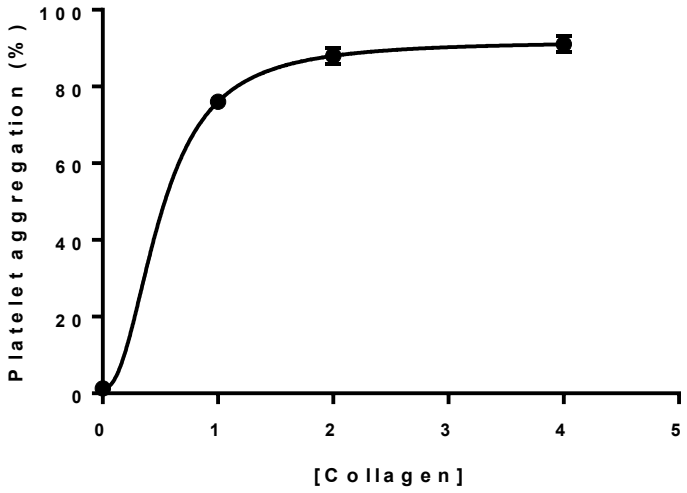

Figure 9.3: Dose response for the collagen in healthy PRP
Maximal platelet aggregation (%) for PRP stimulated with increasing collagen (undiluted) in healthy participants. Curves were fitted by non-linear regression. The EC_{50} was 0.50 µg/ml. Data presented as mean ± SEM (n=4).

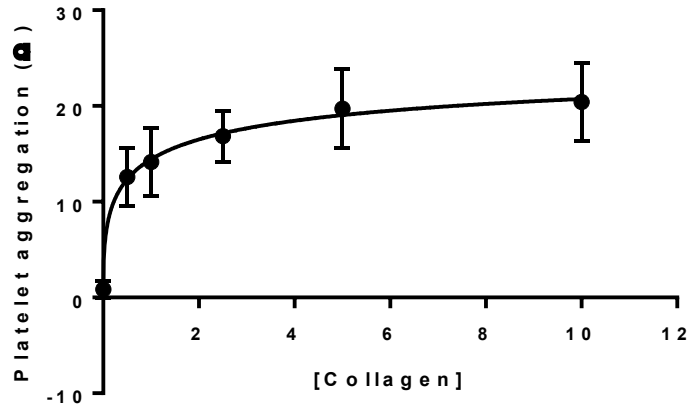

Figure 9.4: Dose response for collagen in healthy whole blood
Maximal platelet aggregation (Ω) for whole blood stimulated with increasing collagen (undiluted) in healthy participants. The EC_{50} was 0.89 µg/ml. Data presented as mean ± SEM (n=7).

Note: The collagen reagent was difficult to dilute because it is an insoluble fibrous protein, therefore the agonist was added to the PRP and whole blood undiluted. For a 500µl PRP sample, an undiluted collagen concentration below 1.0 µg/ml cannot be added due to pipettes having a minimum volume of 0.5 µl. Consequently, it was decided that the minimum volume of 0.5 µl (1.0 µg/ml) would be used for all experiments, including those using PRP from diabetic patients.

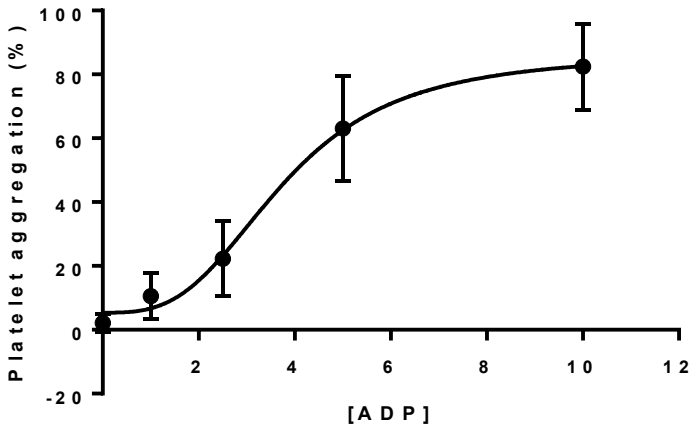

Figure 9.5: Dose response for ADP in diabetic PRP
Maximal platelet aggregation (%) for PRP stimulated with increasing ADP concentrations (μmol/L) in type 2 diabetic participants. Curves were fitted by non-linear regression. The EC_{50} was 3.77 μmol/L. Data presented as mean ± SEM (n=10).

Note: the type 2 diabetic patients had a lower response to ADP than the healthy participants (possibly due to the statin medication) and 2.5 μmol/L did not induce secondary aggregation in the majority of patients, so 5.0 μmol/L ADP was used for all experiments using diabetic blood.

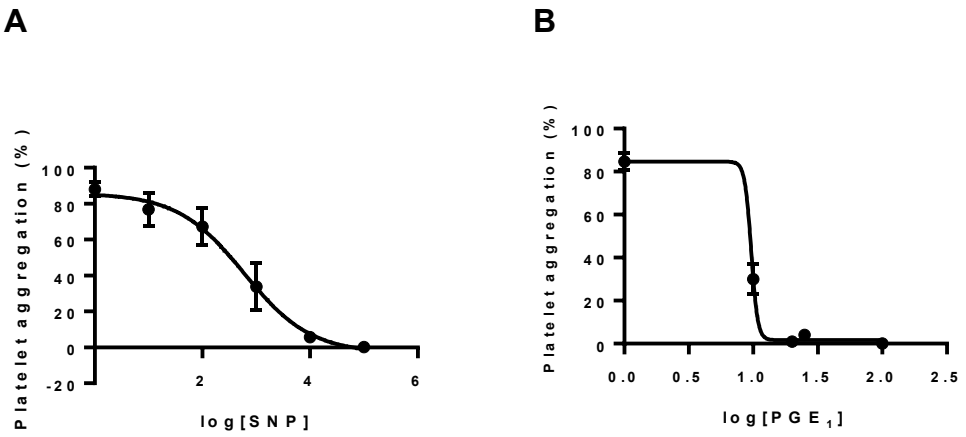

Figure 9.6: Dose response for SNP and PGE_1 in ADP-activated platelets
Figure A represents the dose response for SNP in ADP-activated platelets. The EC_{90} was 135.89 µmol/L (n=4). Figure B represents the dose response for PGE_1 in ADP-activated platelets. The IC_{90} was 0.35 µmol/L. Data presented as mean ± SEM (n=3).

9.2 Standard Curve for ox-LDL ELISA

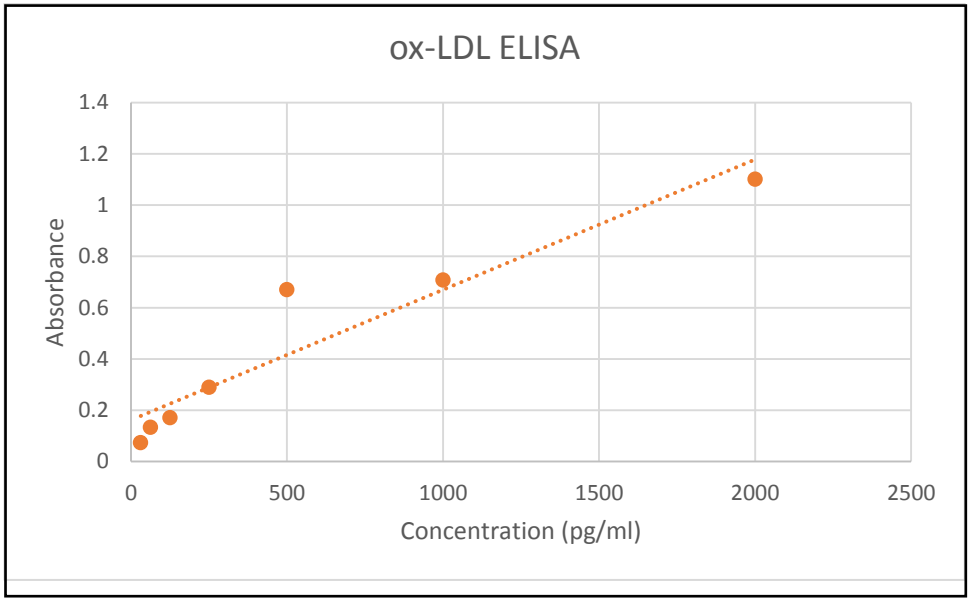

Figure 9.7: Standard curve for ox-LDL ELISA
Standard curve for the ox-LDL ELISA used to determine the ox-LDL concentration (pg/ml) for the EDTA plasma samples.

9.3 Ethical Documentation

9.3.1 Participant consent form (MMU)

All participants recruited for the data for chapter 3 were given a patient consent form before giving blood (see below):

Version 1 — 4th November 2014

Manchester Metropolitan University

Participant Consent Form

Title of Study: Investigating factors that affect thrombosis and haemostasis

Please initial box

1. I confirm that I have read and understand the information sheet dated 10th March 2014 (Version 1) for the above study. ☐

2. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

4. I have received sufficient information about this study ☐

5. I agree to take part in the above study. ☐

_____ _____ _____
Name of participant Date Signature

_____ _____ _____
Name of witness Date Signature

When completed, 1 copy for participant; 1 (original) for researcher site file

9.3.2 Medical screening questionnaire (MMU)

All participants were given a medical screening questionnaire to prevent risk of infection and to ensure that no medication will affect the platelet function assays (see below):

Participant number:

Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This is to ensure that the study protocol will not exacerbate any existing conditions of the participant or put researchers at risk of infection. Please answer the following questions as accurately as possible.

Have you ever suffered from a cardiovascular problem?

i.e. high blood pressure, anaemia, heart attack etc YES/NO

Have you ever suffered from a neurological disorder?

i.e. epilepsy, convulsions etc YES/NO

Have you ever suffered from an endocrine disorder?

i.e. diabetes etc YES/NO

Do you suffer from any allergies?

i.e. any medications, foods etc YES/NO

If you have answered "yes" to any of these questions, please provide details below:

Answering yes to any of the following questions, will exclude you from participation in the study:

Are you currently taking any of the medications/supplements listed below?

- Anti-clotting medications
  - Warfarin
  - Clopidogrel, Ticagrelor, Prasugrel
  - Aspirin
- St John's Wort
- Ginko Bioloba
- Asian Ginseng
- Anti-depressant medication

Have you ever had a positive test for?

HIV

Hepatitis

A bleeding condition or blood disease

Have any of your relatives had Creutzfeld Jacob Disease?

YES/NO

9.3.3 Cover letter: Hyperlipidaemic group

Below is the cover letter that was posted to patients before their appointment at the Lipids Clinic in the MRI. It was posted together with the PIS > 24 hours prior to the patient having blood taken for the study.

Manchester Metropolitan University
Faculty of Science and Engineering
John Dalton East
Chester Street
Manchester
M1 5GD
sarah.daniels2@stu.mmu.ac.uk
Tel: 07801574712

Date XXXXXX

Volunteers for a study to investigate cardiovascular disease in diabetes and hyperlipidaemia

CMFT study number: R01789

Study title: *Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test*

Dear XXXX,

Central Manchester University Hospitals NHS Foundation Trust (CMFT) and Manchester Metropolitan University are investigating factors that affect cardiovascular disease in Type 2 diabetic and hyperlipidaemic patients by analysing a small amount of blood collected from both groups of patients.

In order to recruit participants for this ground-breaking study, we are writing to patients who will shortly be attending the Lipids Clinic. We have enclosed a Patient Information Sheet in order to give potential participants time to read the information and decide whether they would be happy to provide a small blood sample at the Lipids Clinic as part of their assessment.

If you are interested in participating in the study, please email or phone/text Sarah Daniels (sarah.daniels2@stu.mmu.ac.uk or tel:07801574712) stating your name and date of visit. You will then sign a consent form on the day of your appointment. Please note that not all participants who consent to the study will be selected on any one visit to the centre.

The study is part of a larger study at the hospital, consequently, the Patient Information Sheet contains details about other assessments that may, or may not, be undertaken while you are attending the Lipids Clinic. This letter however, specifically relates to the permission to **obtain a blood sample** for a specific aspect of the wider study.

If you have any questions regarding this study, please do not hesitate to contact me.

Yours Sincerely,

Sarah Daniels
Cardio Vascular Trials Unit
Central Manchester University Hospitals NHS Foundation Trust (CMFT) / Manchester Metropolitan University

9.3.4 Patient Information Sheet: Control group

Below is the PIS used for healthy and hyperlipidaemic participants. PIS's were posted or emailed to all patients/healthy subjects at least 24 hours prior to blood being taken for the study.

Central Manchester University Hospitals NHS
NHS Foundation Trust

Study Title

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

- You are being invited to take part in a research study to look at cholesterol (fat) metabolism and cardiovascular health.
- This sheet provides you with the information about the study and how it involves you.
- Before you decide it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully before deciding on whether to take part or not.

Introduction

Changes to the levels of cholesterol (a type of fat) in the bloodstream have profound effects on the health of the heart and blood vessels. Some forms of cholesterol are beneficial to the body while others are harmful. The mechanisms by which these effects occur are not clear. One of the enzymes which can increase the effectiveness of the good cholesterol is called Paraoxonase 1 (or PON1).

In this study, we try to determine which factors affect PON1 activity in patients with diabetes and patients undergoing tests for diabetes.

What is the purpose of this study?

The purpose of this study is to relate changes of PON1 activity with function of blood vessels in subjects with diabetes and subjects undergoing tests for diabetes.

Why have I been chosen?

It is essential that we compare diabetic patients with healthy subjects to help us assess the results. You have been chosen because you are healthy and do not have heart disease and diabetes.

What will I have to do if I take part?

If you agree to take part, we will confirm that you have understood the study and that you meet with the study criteria. You will be asked to sign a consent form for the study and you will need to attend Central Manchester University Hospitals NHS Trust for 1 visit.

What will I have to do if I take part?

During this visit, we will review your medical and medication history. You will be asked to attend for the visit having fasted overnight (for at least 10 hours) so that fasting blood samples can be taken from you. A total of 60ml (about 12 teaspoons) of blood will be taken from you. Most of the blood samples will be analysed in the Manchester laboratory. A small sample of serum or plasma will be retained for future research at the end of the study. In addition, a small frozen anonymised blood sample will be sent to laboratories in Switzerland or Australia for further tests. The tests to be carried out in these laboratories are for research and not for clinical / diagnostic purposes. The tests will provide more information on the relationship between fat metabolism and diabetes. When all the analyses are completed, the samples will be destroyed. You may be asked to give a urine or stool sample for analysis.

What are the possible risks of taking part?

The blood samples will be taken by an experienced doctor or nurse and the only risk involved may be some bruising at the puncture site.

Are there any possible benefits?

There are no immediate benefits to you. However, the knowledge gained from this study will improve our knowledge of factors leading to heart disease and develop new therapies to prevent it.

Will I be paid for taking part in the study?

No. But your travel expenses will be reimbursed. You have the option of receiving £30 as a single payment for each visit, or you can be reimbursed at each visit on the production of taxi receipt for attending and we will arrange a taxi (paid for by the research team) for your return after your visit.

Do I have to take part?

No, taking part is entirely voluntary. If you do not wish to take part you do not have to give a reason.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the researchers. We would ask for your permission to inform your GP of any clinically relevant abnormalities identified during the study.

Complaints

If you have any concerns about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or the Patient Advisory Liaison Service (PALS). Details can be obtained from the hospital.

What do I do now?

If you have any questions please contact:

- Dr Safwaan Adam, Cardiovascular Research Group, University of Manchester, Core Technology Facility (3rd floor), 46 Grafton Street, Manchester, M13 9NT). Tel: 07723526324.

Thank you for taking the time to read this and considering taking part in our research. Please discuss this information with your family, friends or GP, if you wish. You will have at least 24 hours to read this information leaflet. After this time, we will contact you again to see if you are still interested in taking part.

9.3.5 Consent form: Control groups

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

To be completed by the patient: **Please initial the boxes**

1. I confirm that I have read and understood the patient information sheet [version 4 03.05.2016] provided for the study and I have had opportunity to ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to serum and plasma being retained and stored as a gift to the research team and used for future ethically approved research at the end of the study. ☐
5. I consent to my general practitioner being informed of my participation in the study and of any clinically relevant information. ☐
6. I agree for my anonymised blood samples to be transferred to Australia for research purposes. ☐
7. I give my consent to take part in the above study including:
   a. Blood tests ☐
   b. Stool and urine samples ☐

Name……. Date of Birth…….

Signature…… Date……..

I confirm that I have fully explained and discussed with the patient the nature and purpose of the above study.

Name……..Position…… (e.g. Investigator)

Signature…… Date…….

Signature of physician if consent was witnessed by a nurse……

9.3.6 Cover letter: diabetic patients

Below is the cover letter posted with PIS to all type 2 diabetic patients >24 hours prior to blood being taken.

Manchester Metropolitan University
Faculty of Science and Engineering
John Dalton East
Chester Street
Manchester
M1 5GD
sarah.daniels2@stu.mmu.ac.uk

date XXXXXX

Volunteers for a study to investigate cardiovascular disease in Type 2 diabetes
CMFT study number: R01789

Study title: *Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test*

Dear XXXX,

Central Manchester University Hospitals NHS Foundation Trust (CMFT) and Manchester Metropolitan University are investigating factors that affect cardiovascular disease in Type 2 diabetic patients by analysing a small amount of blood collected from diabetic patients.

In order to recruit participants for this ground breaking study, we are writing to patients who will shortly be attending the Diabetes Centre. We have enclosed a Patient Information Sheet in order to give potential participants time to read the information and decide whether they would be happy to provide a small blood sample at the Diabetes Centre as part of their assessment.

If you are interested in participating in the study, please email Sarah Daniels (sarah.daniels2@mmu.ac.uk) stating your name and date of visit. You will then sign a consent form on the day of your appointment. Please note that not all participants who consent to the study will be selected on any one visit to the centre.

The study is part of a larger study at the hospital, consequently, the Patient Information Sheet contains details about other assessments that may, or may not, be undertaken while you are attending the Diabetes Centre. This letter however, specifically relates to the permission to obtain a blood sample for a specific aspect of the wider study.

If you have any questions regarding this study, please do not hesitate to contact me.

Yours Sincerely,

Sarah Daniels
Cardio Vascular Trials Unit
Central Manchester University Hospitals NHS Foundation Trust (CMFT) / Manchester Metropolitan University

9.3.7 Patient Information sheet: Diabetic group

Patient information sheet (PIS) used for diabetic participants. PIS's were posted to all patients >24 hours prior to blood being taken.

Central Manchester University Hospitals NHS
NHS Foundation Trust

Study Title

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

- You are being invited to take part in a research study looking at cholesterol (fat) metabolism and cardiovascular health.
- This sheet provides you with the information about the study and how it involves you.
- Before you decide it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully before deciding on whether to take part or not.

Introduction

Changes to the levels of cholesterol (a type of fat) in the bloodstream have profound effects on the health of the heart and blood vessels. Some forms of cholesterol are beneficial to the body while others are harmful. The mechanisms by which these effects occur are not clear. One of the enzymes which can increase the effectiveness of the good cholesterol is called Paraoxonase 1 (or PON1).

In this study, we try to determine which factors affect PON1 activity in patients with diabetes and patients undergoing tests for diabetes.

What is the purpose of this study?

The purpose of this study is to relate changes of PON1 activity with function of blood vessels in subjects with diabetes and subjects undergoing tests for diabetes.

Why have I been chosen?

You have been chosen because you have either Type 1 or Type 2 diabetes, or because you are undergoing tests to check for diabetes.

What will I have to do if I take part?

If you agree to take part, we will confirm that you have understood the study and that you meet with the study criteria. You will be asked to sign a consent form for the study and you will need to attend Central Manchester University Hospitals NHS Trust for 1 visit only.

You will be asked to attend the visit having fasted overnight (at least 10 hours). This is not essential if you are prone to hypoglycaemia. During the visit, we will review your medical and medication history. You will have a brief physical examination that includes measurements of height, weight, waist circumference and blood pressure. We will perform an ECG test to assess the health of your heart.

A blood sample of 60ml (about 12 teaspoons) will be taken from you. Most of the blood samples will be analysed in the Manchester or Salford laboratory. A small sample of serum or plasma will be retained for future research at the end of the study. In addition, a small frozen anonymised blood sample may be sent to laboratories in Switzerland or Australia for further tests. The tests to be carried out in these laboratories are for research and not for clinical / diagnostic purposes. You will be asked to give a sample of urine for analysis. We may also ask you to provide a stool sample.

The tests will provide more information on the relationship between fat metabolism and diabetes. When all the analyses are completed, the samples will be destroyed.

If you have Type 1 diabetes you will also be invited to have an ultrasound scan looking at the main blood vessels in your neck.

Subjects having an oral glucose tolerance test (OGTT) will be given a sugar drink, and further blood samples will be taken 30 minutes, 2 hours and 4 hours after the drink.

Nerve Function Tests consist of:

- Short questionnaire on pains (if any) in your legs
- Nerve conduction study: Nerves in your legs are stimulated resulting in momentary muscle twitching. This may cause minor fleeting discomfort. Your ability to sense different temperatures and vibration in your lower legs will be measured.
- Corneal sensitivity is assessed with an air puff stimulus to the front of your eyes with no direct contact.
- A corneal confocal microscope will be used to examine the number of nerves in the front part of the eye. A drop of anaesthetic is applied to numb the front of the eye. This allows a gel on the lens of the camera to touch the front of the eye for 1-2 minutes whilst we record images of the cornea.

We will also ask your permission to obtain photographs of the blood vessels at the back of your eyes. The photographs of the back of your eye will be taken using a special camera that does not require the use of dilating drops. There will be no direct contact of the camera with your eyes when these photos are obtained.

What are the possible risks of taking part?

The blood samples will be taken by an experienced doctor or nurse and the only risk involved may be some bruising at the puncture site.

Are there any possible benefits?

There are no immediate benefits to you. However, the knowledge gained from this study will help us to understand the factors leading to heart disease in diabetes and develop new therapies for cardiovascular diseases. If the ultrasound scan shows reduced flow in the blood vessels in your neck this would be an important finding and we would arrange specialist follow-up.

Will I be paid for taking part in the study?

No. But your travel expenses will be reimbursed. You have the option of receiving £30 as a single payment for each visit, or you can be reimbursed at each visit on the production

of taxi receipt for attending and we will arrange a taxi (paid for by the research team) for your return after your visit.

Do I have to take part?

No, taking part is entirely voluntary. If you do not wish to take part you do not have to give a reason and in no way will your future treatment be affected.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the researchers. We would ask for your permission to inform your GP of any clinically relevant abnormalities identified during the study.

Complaints

If you have any concerns about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or the Patient Advisory Liaison Service (PALS). Details can be obtained from the hospital.

What do I do now?

If you have any questions please contact the doctor whose clinics you are attending

- Dr Handrean Soran, Consultant Physician, Manchester Royal Infirmary, Oxford road, Manchester, M13 9WL. Tel: 0161 276 4066 (secretary).
- Dr Akheel Syed, Consultant Physician & Endocrinologist, Salford Royal NHS Foundation Trust, Hope Hospital, Stott Lane, Salford M6 8HD. Tel: 0161 789 7373.
- Dr Naveed Younis, Consultant Diabetologist, Wythenshawe Hospital, Southmoor Road. Manchester M23 9LT. Tel: 0161 291 2396.
- Dr Deepak Bhatnagar, Consultant Chemical Pathologist, Royal Oldham Hospital, Rochdale Road, Oldham OL1 2JH. Tel: 01616 278388.
- Prof Satyan Rajbhandari, Consultant Physician, Lancashire Teaching Hospitals, Preston Road, Chorley PR7 1PP. Tel 01257 245028

Thank you for taking the time to read this and considering taking part in our research. Please discuss this information with your family, friends or GP, if you wish. You will have at least 24 hours to read this information leaflet. After this time, we will contact you again to see if you are still interested in taking part.

9.3.8 Consent form: Diabetic patients

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

To be completed by the patient: **Please initial the boxes**

1. I confirm that I have read and understood the patient information sheet [version 6. 03.05.2016] provided for the study and I have had opportunity to ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to serum and plasma being retained and stored as a gift to Manchester University and used for future ethically approved research at the end of the study. ☐
5. I consent to my general practitioner being informed of my participation in the study and of any clinically relevant information. ☐
6. I agree for my anonymised blood samples to be transferred to Australia for research purposes. ☐
7. I give my consent to take part in the above study including:
   a. Blood tests ☐
   b. Stool and urine samples ☐
   c. Oral glucose tolerance test ☐ (Not applicable if you already have diabetes)
   d. Carotid ultrasound ☐
   e. Nerve function tests ☐
   f. Eye blood vessel photographs ☐

Name……. Date of Birth…….

Signature…… Date……..

To be completed by the investigator or physician or nurse taking consent:

I confirm that I have fully explained and discussed with the patient the nature and purpose of the above study.

Name……..Position…… (e.g. Investigator)

Signature…… Date…….

Signature of physician if consent was witnessed by a nurse……

9.3.9 Questionnaire for control participants

Baseline demographics (such as height, weight and medication) were available for diabetic and hyperlipidaemic patients on the hospital databases but unavailable for control participants (recruited for chapter 4) so a questionnaire was given to this cohort.

Participant ID: ……..
QUESTIONNAIRE:

D.O.B. …….
HEIGHT …….
WEIGHT …….

Do you take any medication? Yes / No
If yes, please give details:
……
……
……
……
……
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……
……...

Do have any medical problems? Yes/No
If yes, please give details:
……
……
……
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……
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……
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…….

Have you taken any Aspirin, Ibuprofen or other non-steroidal anti-inflammatory drugs in the last 2 weeks? Yes / No

Do you smoke? Yes / No

9.4 Flow Cytometry Optimisation

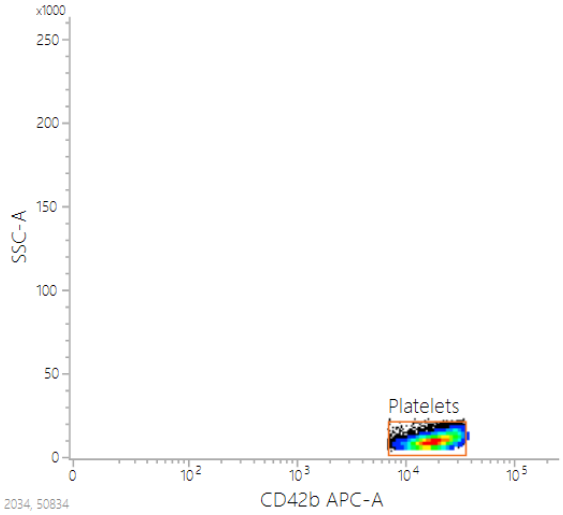

Figure 9.8: Identification of platelets using CD42b
Flow cytometric analysis of human platelets using a APC-conjugated CD42b antibody that recognises an epitope in the GPIbα chain of platelets. Platelet population was gated from 10,000 events positive for CD42b, from a sample of healthy whole blood. Platelets were incubated with CD42b antibody for 20 mins at room temperature before fixation.

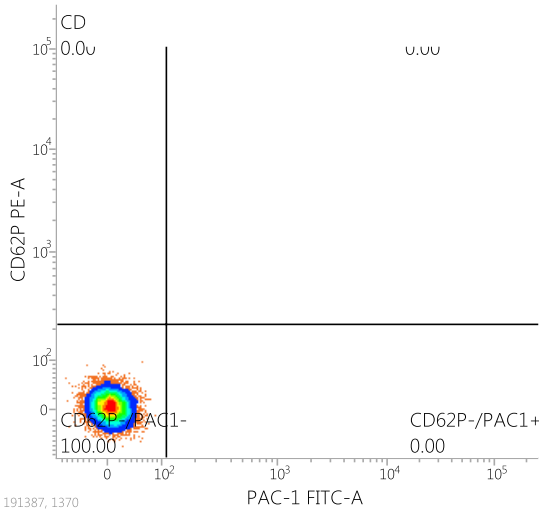

Figure 9.9: Inactivated platelets incubated with CD42b antibody
A dot plot to represent a flow cytometric analysis of human platelets using a CD42b antibody. Platelet population was selected from 10,000 platelets from a sample of whole blood from a healthy participant. Platelets were incubated with CD42b antibody, without agonist, for 20 mins at room temperature before fixation.

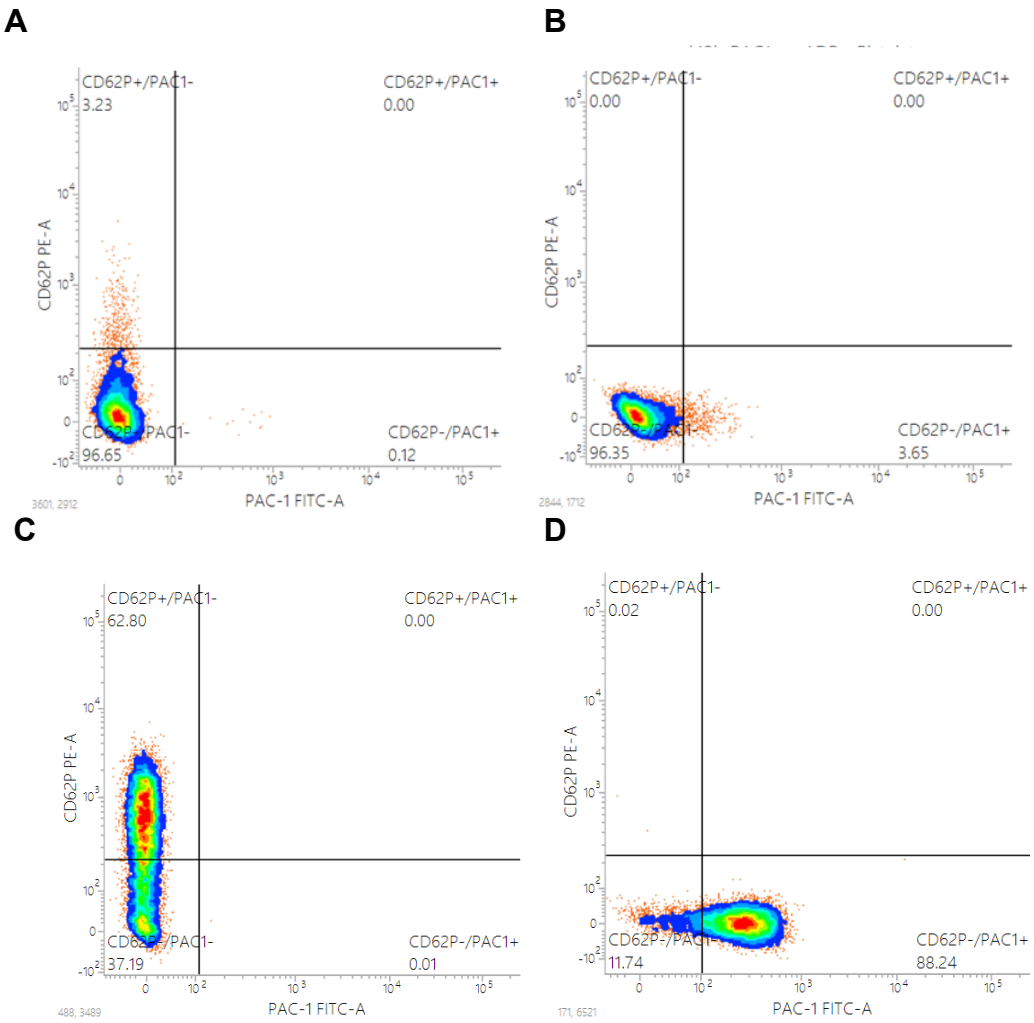

Figure 9.10: Optimisation of the dual-fluorescent assay using CD62P and PAC-1

Optimisation of the flow cytometry assay was performed to avoid bleed-through of PE and FITC fluorochromes in the dual-fluorescent assay. Figures A-D show dot plots to represent flow cytometric analysis of human platelets labelled with CD42b and either CD62P or PAC-1. The platelet population was selected from 10,000 platelets from a sample of whole blood from a healthy participant. Platelets were incubated with antibody for 20 mins at room temperature before fixation. Quadrant gates were set up to divide the populations into $CD62P^-/PAC\text{-}1^-$, $CD62P^+/PAC\text{-}1^-$, $CD62P^-/PAC\text{-}1^+$ and $CD62P^+/PAC\text{-}1^+$. Figure A represents inactivated platelets incubated with CD42b and CD62P. Figure B represents inactivated platelets incubated with CD42b and PAC-1. Figure C represents ADP-activated platelets incubated with CD42b and CD62P. Figure D represents ADP-activated platelets incubated with CD42b and PAC-1.